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<p>(21) International Application Number: PCT/US96/08692</p> <p>(22) International Filing Date: 7 June 1996 (07.06.96)</p> <p>(30) Priority Data: 08/476,864 7 June 1995 (07.06.95) US</p> <p>(71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capitol Square, 400 Locust Street, Des Moines, IA 50309 (US).</p> <p>(72) Inventors: FABIJANSKI, Steven, F.; 6068 Forestglen Crescent, Gloucester, Ontario K1C 594 (CA). ALBANI, Diego; John Innes Centre, Colney Lane, Norfolk NR4 7UJ (GB). LAURIAN, Robert, S.; Plant Research Centre, Central Experimental Farm, Agriculture and AgriFood, K.W. Neatby, 960 Carling Avenue, Ottawa K1A 0C6 (CA). ARNISON, Paul, G.; Farr Biotechnology Group, 1612 Marcoux Drive, Orleans, Ontario K1E 2K6 (CA).</p> <p>(74) Agents: BENT, Stephen, A. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: METHODS AND CONSTRUCTS FOR PRODUCING MALE STERILE PLANTS</p> <p>(57) Abstract</p> <p>A process is described for producing fertile hybrid seed or hybrid seed comprising fertile and sterile seed using male-sterile plants created by employing molecular techniques to manipulate genes that are capable of controlling the production of fertile pollen in plants. Hybrid seed production is simplified and improved by this approach, which can be extended to plant crop species for which commercially acceptable hybrid seed production methods have not been available.</p>		

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Methods and constructs for producing male sterile plants

TECHNICAL FIELD

The present invention relates to a method for producing male sterile plants and hybrid seed, to genetic material used to impart the male sterility trait and to new products produced by said method, namely, genetically transformed plants carrying the male sterile trait, male sterile plants and hybrid seed produced by pollinating said plants with pollen from male fertile plants. The present invention also relates to a method for producing a plant carrying the male sterile trait, or its seed, produced by pollinating said plant with pollen from genetically transformed male fertile plants that, by virtue of the genetic material used, is resistant to herbicides and, if so desirable, is restored in respect to fertility of the hybrid seed.

BACKGROUND

Production of hybrid seed for commercial sale is a large industry. Hybrid plants grown from hybrid seed benefit from the heterotic effects of crossing two genetically distinct breeding lines. The agronomic performance of this offspring is superior to both parents, typically in vigour, yield, and uniformity. The better performance of hybrid seed varieties compared to open-pollinated varieties makes the hybrid seed more attractive for farmers to plant and thereby commands a premium price in the market place.

In order to produce hybrid seed uncontaminated with selfed seed, pollination control methods must be implemented to ensure cross-pollination and not self-pollination. Pollination control mechanisms can be mechanical, chemical, or genetic.

A simple mechanical method for hybrid seed production can be used if the plant species in question has spatially separate male and female flowers or separate male and female plants. The corn plant, for example, has pollen producing male flowers in an inflorescence at the apex of the plant and female flowers in the axils of

leaves along the stem. Outcrossing is assured by mechanically de-tasselling female plants to prevent selfing.

5 Most major crop plants of interest, however, have both functional male and female organs within the same flower so emasculation is not a simple procedure. It is possible to remove by hand the pollen forming organs before pollen shed. But this form of hybrid seed production is extremely labour intensive and expensive. 10 Seed is produced in this manner if the value and amount of seed recovered warrants the effort.

A second general method of producing hybrid seed is to use chemicals that kill or block viable pollen formation. These chemicals, termed gametocides, are used 15 to impart a transitory male-sterility. Commercial production of hybrid seed by use of gametocides is limited by the expense and availability of the chemicals and the reliability and length of action of the applications. These chemicals are not effective for 20 crops with an extended flowering period because new flowers will be produced that will not be affected. Repeated application of chemicals is impractical because of costs.

Many current commercial hybrid seed production 25 systems for field crops rely on a genetic method of pollination control. Plants that are used as females either fail to make pollen, fail to shed pollen or produce pollen that is biochemically unable to effect self-fertilization. Plants that are unable (by any, or 30 a combination, of several different means) to self pollinate biochemically are termed self-incompatible. Difficulties associated with the use of self-incompatibilities are: availability and propagation of the self-incompatible female line and stability of the 35 self-incompatibility. In some instances self-incompatibility can be overcome chemically or immature buds can be pollinated by hand before the biochemical mechanism that blocks pollen is activated. Self-

incompatible systems that can be deactivated are often very vulnerable to stressful climatic conditions that break or reduce the effectiveness of the biochemical block to self-pollination.

5 Of more widespread interest for commercial seed production are systems of pollen control based on genetic mechanisms causing male sterility. These systems are of two general types: (a) genic male sterility, which is the failure of pollen formation because of one or more
10 nuclear genes or (b) cytoplasmic-genetic male sterility (commonly called cytoplasmic male sterility or CMS) in which pollen formation is blocked or aborted because of a defect in a cytoplasmic organelle (mitochondrion) (for general discussions on genic sterility, CMS and hybrid
15 formation in plants see Frankel, R., et al., Pollination Mechanisms, Reproduction and Plant Breeding; Springer V., et al., Monographs on Theoretical and Applied Genetics, N.Y, 1977; Edwardson, J.P., Bot. Rev. 36:341-420, 1970).

 Nuclear (genic) sterility can be either dominant or
20 recessive. A dominant sterility can only be used for hybrid seed production if fertility of the hybrid plants is not critical, and if propagation of the female line is feasible, for example, by clonal propagation or by the use of a selectable marker closely linked to the
25 sterility gene.

 Many successful hybridization schemes involve the use of CMS. In these systems, a specific mutation in the cytoplasmically located mitochondrion can, when in the proper nuclear background, lead to the failure of mature
30 pollen formation. In some other instances, the nuclear background can compensate for the cytoplasmic mutation and normal pollen formation occurs. The nuclear trait that allows pollen formation in plants with CMS mitochondria is called restoration and is the property of
35 specific "restorer genes". Generally the use of CMS for commercial seed production involves the use of three breeding lines, the male-sterile line (female parent), a maintainer line which is isogenic to the male-sterile

line but contains fully functional mitochondria and the male parent line.

5 The male parent line may carry the specific restorer genes (usually designated a restorer line) which then imparts fertility to the hybrid seed. For crops such as vegetables, for which seed recovery from the hybrid is unimportant, a CMS system could be used without restoration. For crops for which the fruit or seed of the hybrid is the commercial product then the fertility
10 of the hybrid seed must be restored by specific restorer genes in the male parent or the male-sterile hybrid must be pollinated. Pollination of non-restored hybrids can be achieved by including with hybrids a small percentage of male fertile plants to effect pollination. In most
15 species, the CMS trait is inherited maternally (because all cytoplasmic organelles are inherited from the egg cell only), which can restrict the use of the system.

In a crop of particular interest herein, the oilseed crop of the species *Brassica napus* or *Brassica campestris*, commonly referred to as canola, no commercial
20 hybrid system has been perfected to date. Mechanical emasculation of flowers is not practical for hybrid seed production on any scale. The use of currently available gametocides is impractical because of the indeterminate
25 nature of flower production. Repeated application of chemicals is expensive and the method is prone to contamination with selfed seed.

Genes that result in self-incompatibility are quite widespread in *Brassica* species and self-incompatible
30 hybrid systems have been used for hybrid seed production in vegetables. Major difficulties are associated with the propagation of the female lines and the breakdown of self-incompatibilities under stressful conditions. Adaptation of these systems to *Brassica* oilseeds is
35 restricted by the expense of increasing the female lines and the availability of appropriate self-incompatible genes in the dominant Canola species, *Brassica napus*.

A variety of sources of male sterility are available in *Brassica* species. Both recessive and dominant genic systems have been reported, however their use is restricted because large scale in vitro propagation or roguing of female lines is in most cases impractical for large scale seed production.

Additionally, a number of CMS systems have been reported in *Brassica* species. Four of these systems have been explored as possible vehicles for hybrid seed production: pol, nap, anand and ogu. The Polima system (pol) has been widely studied and is probably the closest to commercial use. Good restoration and maintenance of pol CMS has been achieved, however the system suffers from potential instability of the CMS with high temperature, a reduction in the heterotic effect of crossing different lines (because of the defective mitochondria) and a reduction in hybrid seed oil content. The use of other CMS systems is also restricted by heat sensitivity (nap), difficulty in restoration of fertility (ogu, anand), difficulty in the maintenance of the sterility (nap) and low temperature chlorosis associated with the sterile cytoplasm (ogu). Improvement of these systems is the object of considerable research, however all of the systems have some inherent weaknesses that limit their utility.

For a general discussion of male sterility in *Brassica* see Shiga, T., Male Sterility and Cytoplasmic Differentiation. In Brassica Crops and Wild Allies, Biology and Breeding, Japan Scientific Societies Press, Tokyo pp. 205-221; Thompson, K.F., *Heredity* 29:253-257).

It is recognized that a desirable system for hybrid seed production in any crop would be a form of genic male sterility that could be regulated or overcome to allow male fertility for the propagation or increase of the female lines or to allow fertility in hybrids. This recognition has stimulated research on the use of molecular systems to effect genic male sterility that could be used for hybrid seed formation. In addition,

the advent and widespread application of recombinant DNA techniques may provide a mechanism of introduction of novel DNA sequences into a wide variety of different crop species that is not possible by the limited sexual methods of genetic exchange between different species. 5 A molecular approach has the advantage that the hybridization system can be imposed on all breeding lines or cultivars of any given crop without the need for extensive backcrossing and disruption of established 10 inbred lines leading to the rapid production of male sterile lines with well characterized and superior agronomic performance.

SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention, a recombinant DNA molecule has been provided for use in preparing a male sterile plant of a pollen-bearing species that is capable of being transformed. The recombinant DNA molecule comprises

- (A) a first DNA sequence coding for a first gene product that converts a first substance which is present in the plant into a second substance, wherein the first and second substances do not inhibit pollen formation or function,
- (B) a second DNA sequence coding for a second gene product that converts the second substance to a third product that inhibits pollen formation or function,
- (C) a first promoter that controls expression of the first DNA sequence in plants,
- (D) a second promoter that controls expression of the second DNA sequence in plants, and
- (E) a third DNA sequence that confers resistance to a selective agent.

In a preferred embodiment, the aforementioned resistance is to a herbicide, such as a herbicide selected from the group consisting of glyphosate, glyphosinate and TBL.

In another preferred embodiment, at least one of the first and second promoters is specific to cells that are critical to pollen formation or function. In particular, either or both of the first and second promoters can be specific to anther cells or, alternatively, to microspores. In still another preferred embodiment, the second promoter is an inducible promoter.

In a recombinant DNA molecule as described above, the first DNA sequence can be IamS and second DNA sequence IamH. Alternatively, the second substance and the second expression product can be paired as follows: 2-amino-4-methoxy-butanoic acid and methoxinine dehydrogenase or rhizobitoxine synthase; a non-toxic analog of glucuronic

acid and β -glucuronidase; naphthalene acetamide or indole acetamide and indole acetamide hydrolase.

5 In accordance with another aspect of the present invention, there is provided a method for producing a male sterile plant, comprising the steps of

(A) introducing a recombinant molecule as described above into the genome of a pollen producing plant which is capable of being genetically transformed; and (B) growing the pollen producing plant under conditions such that
10 male sterility is achieved as a result of the expression of the first DNA sequence and the second DNA sequence, providing the male sterile plant. In a preferred embodiment, the method further comprises, after step (B), the steps of (C) crossing the male sterile plant with an
15 fertile, isogenic plant to obtain seed, then (D) growing out the seed to produce progeny plants, and (E) selecting male sterile plants from among the progeny by exposure to the selective agent.

20 According to yet another aspect of the present invention, a method is provided for producing hybrid seed from a male sterile plant, comprising the steps of

(A) introducing a first recombinant molecule as described above into the genome of a pollen producing plant which is capable of being
25 genetically transformed;

(B) growing the pollen producing plant under conditions such that male sterility is achieved as a result of the expression of the first DNA sequence and the second DNA sequence, yielding a male sterile plant;
30 and

(C) crossing the male sterile plant with pollen from a male fertile line, the pollen having integrated into its genome a second recombinant DNA molecule comprising a selective marker gene and a promoter
35 regulating expression of the selective marker gene in plants.

Pursuant to a preferred embodiment of this method, the second recombinant DNA molecule further comprises

(i) a third DNA sequence and (ii) a third promoter that causes transcription of the third DNA sequence in plants, wherein the third DNA sequence is transcribed as an antisense RNA or encodes a gene product that negates the effect of the first DNA sequence or the second DNA sequence, such that hybrid seed with restored fertility is produced by the male sterile plant, and the third promoter causes the transcription such that expression of the third DNA sequence overlaps in time with expression of the first DNA sequence or the second DNA sequence. In the alternative, the third DNA sequence can have an antisense RNA transcript, can be a repressor gene, or can encode a gene product that is a ribozyme, a dominant negative transactivator, or a transdominator

According to another preferred embodiment, the selective marker genes imparts herbicide resistance.

Various products also come within the scope of the present invention. These include, for example, hybrid seed produced by a method described above, a plant cell comprising elements (A) through (E) of the aforementioned recombinant DNA molecule, and a differentiated plant comprising such a cell.

The following advantages over other hybridization systems may be obtained with the present invention:

(a) Hybrid seed production is not labour intensive and can be achieved on a large scale with commercially acceptable costs.

(b) Male sterility is simply inherited and stable in response to environmental stresses that limit the effectiveness of self-incompatibility and CMS based schemes.

(c) Seed that is produced will be relatively uncontaminated by selfed seed.

(d) The system avoids the use of defective cytoplasmic organelles that may detract from the performance of hybrid seed.

(e) The system will greatly speed the development and increase the number of lines that can be tested as

parents in a hybrid cross because it can be imposed on any plant or inbred line capable of being transformed and regenerated into plants without the inclusion of additional genomic DNA. Additionally plant lines can be
5 tested for combining ability before inclusion of the hybridizing system which can modify breeding strategy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of the construction of an anti-sense gene vector that was used for anti-sense
10 RNA inhibition of β -glucuronidase gene activity in transgenic plants.

Figure 2a is a schematic representation of a restriction map and coding region of clone number L 4, a microspore specific clone isolated from a *Brassica napus*
15 genomic library. The clone contains three similar copies of a single gene. These genes are identified as Bp4A, Bp4B and Bp4C. The first (Bp4A) and third (Bp4C) genes are functional, the second gene has modifications that most likely render it non-functional. The restriction
20 map is made diagrammatically in that the non-transcribed regions are shown as a single line, while the transcribed regions are shown as a boxed area. The second gene (Bp4B) is identified on the basis of sequence homology and is shown as a boxed area with a dotted line. The
25 notation "del 220" refers to an approximately 220 base pair deletion/rearrangement that has presumably inactivated the second gene (Bp4B) in this clone. Start of transcription is located at the leftmost side of each boxed area (except in the case of gene Bp4B) and exon and
30 intron positions are noted by the exons being filled in with black and the intron positions being left unfilled. A small arrowhead is shown on the non-transcribed 5' region of each gene, this arrowhead serves to indicate the promoter region of each gene. Restriction sites are
35 identified such that the number of the first nucleotide of the restriction enzyme recognition site is shown. Not all restriction sites are shown, only those relevant to

the constructs detailed within are indicated. The genes are presented with the 5' region on the left side and the 3' region on the right side. The numeration of the DNA sequence is from left to right, 5' to 3' in all cases.

5 Figure 2b is a schematic representation of a restriction map and coding region of clone number L 10, a microspore specific clone isolated from a *Brassica napus* genomic library. The clone contains a single gene. Start of transcription, exon, intron and promoter positions are noted as in Figure 2a. Restriction sites are identified such that the number of the first nucleotide of the restriction enzyme recognition site is shown. The genes are presented with the 5' region on the left side and the 3' region on the right side. The
10 numeration of the DNA sequence is from left to right, 5' to 3' in all cases.
15

Figure 2c is a schematic representation of the restriction map and coding region of clone number L 16, a microspore specific clone isolated from a *Brassica napus* genomic library. The clone contains a single gene that shows similarity to clone L 10. Intron and exon positions are noted as in Figure 1. Restriction sites are identified such that the number of the first nucleotide of the restriction enzyme recognition site is shown. The genes are presented with the 5' region on the left side and the 3' region on the right side. The
20 numeration of the DNA sequence is from left to right, 5' to 3' in all cases.
25

Figure 2d is a schematic representation of the restriction map and coding region of clone number L 19, a microspore specific clone isolated from a *Brassica napus* genomic library. The clone contains a single gene. Start of transcription, exon, intron and promoter positions are noted as in Figure 1. Restriction sites are identified such that the number of the first nucleotide of the restriction enzyme recognition site is shown. The genes are presented with the 5' region on the left side and the 3' region on the right side. The
30
35

numeration of the DNA sequence is from left to right, 5' to 3' in all cases.

Figure 3a is the complete nucleotide sequence of the clone L 4 represented in Figure 2a. Only the coding strand is shown, for clarity.

Figure 3b is the nucleotide sequence of the portion of the clone L 10 shown in Figure as being underlined in Figure 2b. Only the coding strand is shown, for clarity.

Figure 3c is the nucleotide sequence of the portion of the clone L 16 shown in Figure as being underlined in Figure 2c. Only the coding strand is shown, for clarity.

Figure 3d is the nucleotide sequence of the portion of the clone L 19 shown in Figure as being underlined in Figure 2d. Only the coding strand is shown, for clarity.

Figure 4 is the nucleotide sequence of 3 cDNA clones isolated from a microspore derived cDNA library of *Brassica napus*. These clones are named cBp401, cBp405, and cBp408. These three cDNA clones are extremely homologous to members of the L4 *Brassica napus* microspore specific gene family (Bp4A, Bp4B, Bp4C). The nucleotide sequence of two of these 3 members of the L4 *Brassica napus* microspore specific gene family are shown in this Figure (Bp4A, Bp4C). The gene Bp4C was chosen as a master sequence for comparison. The deduced nucleotide coding sequence for the genes Bp4A and Bp4C is shown as a sequence from which the two exons of the genes have been spliced together at the positions normally spliced in vivo. This gives rise to the coding sequence in the mature mRNA. The cDNA clones are aligned with the sequence of Bp4C such that only nucleotide changes are shown. The sequences are therefore represented as variants of a single master sequence of gene Bp4C which is shown on line 1. The ATG start codon as well as the TGA or TAA stop codons are underlined. These three cDNA clones correspond to related members of the *Brassica napus* microspore specific gene family a portion of which is contained in the clone L4.

Figure 5 shows a partial nucleotide sequence of a cDNA clone that is closely homologous to the gene contained in the clone L10, the restriction map of which is shown in Figure 2b.

5 Figure 6 is the nucleotide sequence of the cDNA clone that is the gene product of clone L19, the restriction map of which is shown in Figure 2d.

10 Figure 7 (7A, 7B, 7C, 7D) are schematic representations describing the production of vectors containing the promoter and promoter regions from clone L4. The specific examples are discussed in greater detail below. Figure 7E is a schematic representation of the promoter constructs produced as shown schematically in Figures 7A to 7D.

15 Figure 8 is a schematic representation describing the production of vectors containing the promoter regions of clone L10, the details of which are discussed below.

20 Figure 9 is a schematic representation describing the production of vectors containing the promoter regions of clone L19, the details of which are discussed below.

25 Figure 10 is a schematic representation of a restriction map of a *Brassica napus* genomic clone that contains a gene (referred to as HP101) that is constitutively expressed at high levels in all cells including developing pollen cells. The portion of the clone that was used to provide promoter regions for the production of anti-sense RNA is shown, this construct gives rise to an anti-sense RNA that contains a region of transcribed RNA from this gene.

30 Figure 11 is a schematic representation describing the production of a gene coding for a polylysine protein.

35 Figure 12 is a schematic representation describing the production of an anti-sense gene specific to the intron region of clone L19 and a restorer that lacks the intron region targeted for anti-sense RNA inhibition.

Figure 13 is a schematic representation describing the production of clones containing versions of a ricin A chain coding region.

Figure 14 is a histogram showing GUS activity in plants transformed with sense and anti-sense GUS genes.

Figure 15 illustrates the procedure used for the isolation of the T-DNA gene 2 (the IamH: indole acetamide hydrolase gene) of the *Agrobacterium tumefaciens* Ti plasmid derivative pPCV 311 (described by Koncz, C. and Schell, J., Molecular and General Genetics, 1986, 204:383-396) and the construction of a promoterless version of this gene.

Figure 16 illustrates the procedure used for the isolation of the T-DNA gene 1 (the IamS: indole acetamide synthase gene) of the *Agrobacterium tumefaciens* Ti plasmid derivative pPCV 311 and the construction of a promoterless version of this gene.

Figure 17 illustrates hybrid seed production using the binary cryptocytotoxicity method based on the use of the IamH and IamS genes described in Figures 15 and 16.

Figure 18 illustrates the segregation patterns of the IamH and IamS genes in the F1 and F2 populations when the genes are on the same segregation unit.

Figure 19 is an alternate representation of the procedure used to isolate the T-DNA gene 2 (the IamH gene) of *Agrobacterium tumefaciens* and the production of a promoterless version of this gene.

Figure 20 illustrates hybrid seed production using a variation of the binary cryptotoxicity method and a herbicide resistance gene. Genes 1 and 2 can be the IamS and IamH genes. The herbicide resistance gene can be any resistance gene against any herbicide. The herbicide resistance gene has a dual role: it results into a hybrid plant and seed protected from herbicide treatment and is essential for selection of parent A when the parent is maintained via a cross with parent A'. The resulting hybrid seed is essentially 100% herbicide resistant and restored in respect to male fertility.

Figure 21 illustrates hybrid seed production with parent A being produced and maintained as shown in Figure 20. Parent B is homozygous to herbicide

resistance. The hybrid is essentially 100% herbicide resistant.

Figure 22a illustrates the use of the binary cryptotoxicity method for production of a male sterile, herbicide resistant parent A. The resulting parent A is essentially 100% male sterile with G1 and G2 on different chromosomes. Parent A is herbicide resistant.

Figure 22b illustrates the method used to maintain parent A shown in Figure 22a. Notice use of herbicide spraying to maintain the male sterile parent A.

Figure 22c illustrates the crossing of parent A shown in Figure 22a with parent B. Parent B is the result of a transformation where the restorer gene and the herbicide resistance gene are linked on one recombinant DNA molecule. The result of crossing parent A and parent B is essentially a hybrid that is restored in regard to male fertility and is herbicide resistant.

Figure 23 is a map of plasmid pPHP5838 which contains the Bp10 promoter operably linked to the IAMS gene and the nos 3' nontranslated region.

Figure 24 is a map of plasmid pPHP5839 which contains the Bp10 promoter operably linked to the IAMH gene and the nos 3' nontranslated region.

Figure 25 is a map of plasmid pPHP5840 which is the TA39(8B3) promoter and the 0' nontranslated leader operably linked to the IAMS gene and the PI-II 3' nontranslated region.

Figure 26 is a map of plasmid pPHP5841 which contains the TA39(8B3) promoter and nontranslated leader operably linked to the IAMH gene and the PI-II 3' nontranslated region.

Figure 27 is a partial sequence of pPHP5838, where the nucleotide coordinates correspond to those used in the map of Figure 23. A few nucleotides are presented in small letters to indicate uncertainty at that point in the sequence.

Figure 28 is a partial sequence of pPHP5839, where the nucleotide coordinates correspond to those used in

the map of Figure 24. A few nucleotides are presented in small letters to indicate uncertainty at that point in the sequence.

5 Figure 29 is a partial sequence of pPHP5840, where the nucleotide coordinates correspond to those used in the map of Figure 25. A few nucleotides are presented in small letters to indicate uncertainty at that point in the sequence.

10 Figure 30 is a partial sequence of pPHP5841, where the nucleotide coordinates correspond to those used in the map of Figure 26. A few nucleotides are presented in small letters to indicate uncertainty at that point in the sequence.

DETAILED DESCRIPTION OF THE INVENTION

15 As hereinbefore mentioned, the invention relates to recombinant DNA molecule(s) for use in the preparation of a plant or seed that are male sterile or carry a male sterile trait. The male sterile plant is created in order to facilitate hybrid crosses. The invention also
20 relates to use of recombinant DNA molecule(s) introduced in the male fertile parent of such a cross, resulting in a hybrid seed restored in respect to fertility.

The recombinant DNA molecule(s) for use to produce male sterility contains one or more DNA sequences which may be
25 the same or different, which encode gene product(s). The gene product(s) include a nucleotide sequence (in particular a RNA sequence), a protein or a polypeptide. When produced in a cell and/or tissue of a plant which is essential to pollen formation and/or function, the gene
30 product is directly or indirectly capable of interfering with the function and/or development of the cell. The presence of the recombinant DNA molecule can be selected if the DNA also carries a selective marker gene, preferably for herbicide resistance. A cell and/or
35 tissue of a plant which is essential to pollen formation and/or function includes cells and/or tissues that are instrumental in the development or function of pollen, including cells and/or tissues from which pollen develops

(e.g., premeiotic and uninucleate microspore cells), cells and/or tissues which form part of the male structure in which pollen develops (e.g., anther, tapetum or filament) and pollen itself. The recombinant DNA molecule may also contain one or more promoters, which may be the same or different, which are capable of regulating the expression of the DNA sequences in the recombinant DNA molecule. The DNA sequences and promoters in the recombinant DNA molecule are selected such that the gene product selectively interferes with the function and/or development of a cell of a plant that is essential to pollen formation and/or function.

The recombinant DNA molecule of the invention may contain one or more DNA sequences which may be the same or different. The DNA sequences may be selected from the following DNA sequences:

- (a) a DNA sequence which encodes an RNA which when produced in a cell of a plant which is essential to pollen formation and/or function substantially interferes with the expression of;
 - (i) a gene which is essential to pollen formation and/or function;
 - (ii) a gene which is essential to the continued development and/or function of all metabolically competent cells of a plant; or
 - (iii) a gene which confers on a cells of a plant resistance to a chemical agent or physiological stress;
- (b) a DNA sequence which encodes a protein or polypeptide which when produced in a plant cell which is essential to pollen formation and/or function is substantially cytotoxic to the cell; and
- (c) a DNA sequence which encodes a protein or polypeptide which when produced in a plant cell which is essential to pollen formation or

function renders a non-toxic substance substantially cytotoxic to the cell; and

5 (d) a DNA sequence which encodes a gene product which when produced in a plant cell which is essential to pollen formation and/or function renders the cell susceptible to a chemical agent or physiological stress.

10 (e) a DNA sequence which encodes a gene product which may be converted into a substance which is cytotoxic to cells/tissues essential for pollen formation and/or function.

The above DNA sequences (a) to (e) are further described below in relation to the various embodiments of the invention.

15 According to a first embodiment of the present invention genic male sterility may be produced by transforming plant cells that are capable of regeneration into a differentiated whole plant, with a recombinant DNA molecule containing an anti-sense gene which encodes a
20 gene product, in particular RNA, which is capable of interfering with the expression of a targeted gene. Preferably, the recombinant DNA molecule encodes RNA which is complementary to and capable of hybridizing with the RNA encoded by the targeted gene.

25 An anti-sense gene is a DNA sequence produced when a gene is inverted relative to its normal presentation for transcription. An anti-sense gene may be constructed in a number of different ways, provided that it is capable of interfering with the expression of a targeted
30 gene. Preferably, the anti-sense gene is constructed by inverting the coding region of a gene relative to its normal presentation for transcription to allow for transcription of its complement; hence the RNA's encoded by the anti-sense and the targeted gene are
35 complementary. It is understood that a portion of an anti-sense gene incorporated in the recombinant DNA molecule of the invention may be sufficient to selectively interfere with the expression of a gene and

thus the term "anti-sense gene" used herein encompasses a functional portion of an anti-sense gene.

5 The gene targeted for inactivation is a gene expressed in cells and/or tissues of a plant that are essential to pollen formation and/or function. Preferably, the gene is expressed only at specific stages during pollen development whose regulation is tightly controlled. The gene may also be a gene that is essential for the continued development or function of
10 all metabolically competent cells/tissues such as but not limited to genes involved in essential cellular structures, and essential metabolism including essential biosynthesis. The gene further may be a gene which confers on cells/tissues of a plant resistance to a
15 chemical agent or physiological stress.

It is understood herein that it is not essential to identify and isolate the target gene *de novo*. Rather, the gene may have been described in the literature or obtained commercially.

20 The isolation of genes that are essential to pollen formation and/or function may be accomplished by a variety of procedures. A detailed description of a procedure for isolating such genes is set out below.

Since the gene in question may initially be isolated
25 in its native form, it is to be understood that the term "gene" as used herein may refer to one or more parts of the gene including 5' untranslated leader sequences, sequences functional in ribosome attachment, coding sequences, promoter sequences, intron sequences, and
30 untranscribed 3' sequences, or any substantial fragments of these sequences.

The present inventors have isolated DNA sequences from a plant of the species *Brassica napus ssp oleifera* w Westar which are expressed only in microspores and
35 whose expression is essential to microspore function and/or development and hence a preferred embodiment of the present invention provides a recombinant DNA molecule containing one or more anti-sense genes to these isolated

DNA sequences. A schematic representation of the restriction maps and coding regions of the microspore specific genes identified as L4, L10, L16 and L19 are shown in Figures 2a, 2b, 2c, and 2d, respectively. The complete nucleotide sequence of clones L4, and relevant sequences of L10, L16 and L19 are shown in Figures 3a, 3b, 3c, and 3d, respectively. The nucleotide sequences of isolated cDNA clones that correspond to the genes or related gene family members within clones L4, L10 and L19 are shown in Figures 4, 5, and 6, respectively. In a preferred embodiment of the invention the recombinant DNA molecule contains one or more of an anti-sense gene to the microspore specific genes identified as L10 and L19.

It is believed that homologous microspore specific DNA sequences to those isolated from *Brassica napus* described herein will be found and expressed exclusively in pollen of other species of pollen-bearing plants, particularly species of plants within the genus *Brassica* and the family *Cruciferae* (also known as *Brassicaceae*), and more particularly other cultivars of *Brassica napus*.

The occurrence of the microspore specific sequences isolated from *Brassica napus* in other species of pollen-bearing plants may be routinely ascertained by known hybridization and DNA polymerase extension techniques. It is believed that the similarity of plant genes from species to species will allow for the embodiments of the present invention to be carried out using the DNA sequence in any number of pollen bearing plant species that are capable of being genetically transformed. The universality of plant genes has been widely documented in the literature and homologous plant genes have been described for plant actins (Shah, D.M., et al., J. Mol. Appl. Genet. 2:111-126, 1983), phytochrome (Hershey, H.P., et al., Proc. Natl. Acad. Sci. USA 81:2332-2337, 1984) storage proteins (Singh, N.K., et al., Plant Mol. Biol. 11:633-639, 1988) enzymes such as glutamine synthase (Lightfoot, D.A., et al., Plant Mol. Biol. 11:191-202, 1988, and references within) and nitrate

reductase (Cheng, C., et al., EMBO Jour. 7:3309-3314). These and other examples in the literature clearly demonstrate that many plant genes are highly conserved. It is also clear that this conservation applies not only to structural proteins but to enzymatic proteins important to cellular physiology. Therefore, it is believed that the DNA sequences, when found in another plant species, will be essential to microspore development and will be useful in carrying out the present invention in such species.

It has also been demonstrated that anti-sense RNA sequences derived from one plant species can effectively inhibit the expression of homologous DNA sequences in a different species. Van der Krol, et al., 1988, Nature 333:866-869. Therefore, it is expected that anti-sense RNA derived all or in part from *Brassica* microspore specific DNA sequences will be functional in other plants. Furthermore, the identification of the homologous gene in other plants by the hybridization and/or DNA polymerase extension techniques mentioned above will allow a person trained in the art to, if necessary, design an anti-sense gene which will more precisely complement the targeted gene and be more effective in inactivating such gene.

As hereinbefore mentioned the targeted gene may also be essential for the development and/or function of all metabolically competent cells/tissues such as, but not limited to, genes involved in essential cellular structures, essential biosynthesis and essential metabolism. Examples of such sense genes may be found in the literature and include the genes which encode actin, tubulin or ubiquitin; three proteins which are essential to cellular growth and development.

Sequences for actin genes isolated from plants have been published (for example; Baird W.V., and Meagher, R.B., EMBO J. 6:3223-3231, 1987, or Shah, D.M., Hightower, R.C. and Meagher, R.B., Proc Natl Acad Sci USA 79: 1022-1026, 1982) and actin is known to play a

critical role in normal cellular function especially during mitosis and meiosis where actin forms part of the cellular apparatus for cellular division.

5 The sequence for plant tubulin has also been described (Raha, D., Sen, K. and Biswas, B.B. Plant Mol Biol 9:565-571, 1987). Tubulin, like actin, is known to be important in the cellular life-cycle particularly in regards to cell shape, transport and spindle formation during mitosis and meiosis.

10 The DNA sequence for plant ubiquitin has also been published (Gausling, K. and Barkardottir, R. Eur J. Biochem 158:57-62, 1986). Ubiquitin is a protein involved in the turnover of cellular proteins and as such has a critical role in the regulation of specific
15 cellular protein levels. In addition, ubiquitin is one of the most highly conserved proteins in eukaryotic cells. Interference with ubiquitin expression can cause abnormalities in the turnover of cellular proteins.

20 If any of the aforementioned proteins are either not present, or are present but not in a normal functional form in the cell, proper cellular function is interfered with and the cell fails to develop properly.

25 It is believed that a gene that is found to be essential for the continued development or function of all metabolically competent cells in one plant species will have a similar counterpart in other plant species, since it is generally understood that within the plant kingdom there are genes that are nearly identical or very homologous involved in the basic processes that control
30 or are a result of cellular development. It is further believed that a gene which encodes a gene product which interferes with the expression of the gene (i.e., an anti-sense gene) in one plant species will have the ability to do so in other plant species.

35 The tissue-specific and developmentally regulated expression of a wheat endosperm protein synthesized in tobacco plants genetically transformed with this wheat gene has been reported (Flavell, R.B., et al., Second

International Congress for Plant Molecular Biology, Abstract #97). In that example, the wheat gene functioned in the tobacco plant in an identical fashion to the way in which it functions in a wheat plant. Other literature clearly shows that the regulation of a specific gene, which can be in many cases complex, is maintained in transgenic plants. One example of this is the phytochrome mediated regulation of a wheat Chlorophyll a/b-binding protein in transgenic tobacco (Nagy, F. et al., EMBO Jour. 5:1119-1124, 1986). In this example the light responsive specific regulation of the wheat gene was maintained in the foreign genetic environment. Not only do cereal genes function in a conserved manner, but genes from other plant species that are more closely related maintain functionality in heterologous genetic systems. Pea seed proteins are expressed properly in tobacco plants (Higgins, T.J.V., et al., Plant Mol. Biol. 11:683-696, 1988), as are soybean seed proteins, (Barker, S.J., et al., Proc. Natl. Acad. Sci.USA 85:458-462, 1988) and pea rbcS genes (Nagy, F. et al., EMBO Jour. 4:3063-3068, 1985). The scientific literature has numerous other examples of genes that have been used to genetically transform plants and those genes maintain their ability to function properly in this new genetic environment. Therefore the conserved nature of these genes, not only in the DNA sequences which control the expression of these genes, but the actual protein structure coded for by these genes, is similar among the plant species.

As hereinbefore mentioned, the targeted gene may be a gene which confers on cells of a plant resistance to a chemical agent or a naturally occurring or artificially incurred physiological stress. Such a gene may be native to a plant cell or may be a foreign gene, for example, a gene from another plant species. If the gene is a foreign gene it may be introduced into the genome of a plant cell prior to, after, or concurrently with the anti-sense gene as will be described below. Examples of

such genes may be identified in the literature and include genes which confer resistance to a herbicide such as acetolactate synthase which confers resistance to chlorosulfuron; genes which confer resistance to a physiological stress such as the gene encoding superoxide dismutase which actively reduces oxygen radicals; genes which confer resistance to uv light and wounding (phenyl and alanine ammonia lyase or chalcone synthase); genes which confer resistance to disease or pests (e.g., β 1,3 glucanases, chitinase or proteinase); genes which confer resistance to antibiotics and certain toxic drugs such as the gene encoding neomycin phosphotransferase II conferring resistance to kanamycin.

The recombinant DNA molecule containing an anti-sense gene of the present invention additionally contains a promoter which regulates the expression of the anti-sense gene. If more than one DNA sequence containing an anti-sense gene (or one or more of a gene encoding a cytotoxic substance or a gene which confers susceptibility to a chemical agent or physiological stress) is present in the recombinant DNA molecule of the invention, the DNA sequences may be regulated by an identical promoter or each may be regulated by a different promoter.

If the targeted gene is a gene which is only expressed in cells/tissues of a plant that are essential to pollen formation and/or function, then a promoter that functions in all, many, or a variety of cell types including cells/tissues essential to pollen formation and/or function may be used in the construction of an anti-sense gene. An example of such a constitutive promoter is CaMV 35S or preferably HP 101 which has been isolated from *Brassica napus* as described below. Thus, the promoter that is used may be constitutively active in all or many cell types but the anti-sense gene whose expression is regulated by such a promoter will only interfere with the expression of the targeted gene in those cells/tissues producing the targeted gene in

question, i.e., cells/tissues essential to pollen formation and/or function.

It is also possible to use an inducible promoter to regulate the expression of the anti-sense gene. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically, the protein factor that binds specifically to an inducible promoter to activate transcription is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer may be a chemical agent such as a protein, metabolite (sugar, alcohol etc.), a growth regulator, herbicide, or a phenolic compound or a physiological stress such as heat, salt, toxic elements etc. or the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell such as by spraying, watering, heating, or similar methods. Examples of inducible promoters include the inducible 70 KD heat shock promoter of *D. melanogaster* (Freeling, M., Bennet, D.C., Maize ADN 1, Ann. Rev. of Genetics 19:297-323) and the alcohol dehydrogenase promoter which is induced by ethanol (Nagao, R.T., et al., Mifflin, B.J., Ed. Oxford Surveys of Plant Molecular and Cell Biology, Vol. 3., p. 384-438, Oxford University Press, Oxford 1986). The inducible promoter may be in an induced state throughout pollen formation or at least for a period which corresponds to the transcription of the sense gene. A promoter that is inducible by a simple chemical is particularly useful since the male sterile plant can easily be maintained by self-pollination when grown in the absence of such a chemical.

Alternatively, if the targeted gene is a gene essential for the development and/or function of all metabolically competent cells/tissues or a gene which

confers on cells of a plant resistance to a chemical agent or physiological stress, a promoter that is active only in cells/tissues essential to pollen formation and/or function (for example, a promoter essential for pollen development and/or function) is preferably used to regulate the expression of the anti-sense gene.

The invention also relates to a promoter essential for pollen development and/or function or functional portions of the promoter thereof and chimeric promoters constructed using the promoter essential for pollen development and/or function and/or portions thereof.

A promoter essential for pollen development and/or function is a DNA sequence which regulates the expression of a DNA sequence selectively in the cells/tissues of a plant essential to pollen formation and/or function and/or limits the expression of such a DNA sequence to the period of pollen formation in the plant.

Preferably, the promoter essential for pollen development and/or function is a DNA sequence corresponding to the promoter sequence in the microspore specific genes identified as L4, L10, L16 and L19 herein or a functional fragment thereof; or a chimeric promoter sequence containing one or more of a promoter sequence from the microspore specific genes identified as L4, L10, L16 and L19 or portions of such promoter sequences. The preferred promoter essential for pollen development and/or function may be used in conjunction with naturally occurring flanking coding or transcribed sequences described herein or with any other coding or transcribed sequence that is essential to pollen formation and/or function.

The promoters essential for pollen development and/or function referred to above were isolated from a plant of the species *Brassica napus*. It is believed that it is possible to use these promoters to limit the expression of a given DNA sequence to pollen formation and/or function and to a specific period during pollen

formation and/or development from a plant of a different species. The published scientific literature has clearly shown that plant genes are universal and the plant tissue-specific promoter fragments retain their function in other species. For example, wheat endosperm promoter fragments function to give appropriate seed specific expression in tobacco (Simpson, J. et al., EMBO Jour. 4:2723-2729, 1985) and the alcohol dehydrogenase promoter (Adh-1) from corn (Zea mays) can be used in conjunction with other promoter fragments to give appropriate expression in tobacco (Ellis, J. G., et al., 1987, EMBO J. 6: 11-16). Additionally the maize transposable element Ac is active in tobacco and other plant species (Taylor, et al., 1989, Plant Mol. Biol. 13: 109-118) providing further evidence of the universality of plant gene structure and function. These examples demonstrate the equivalent tissue specific function of the same promoters in very widely divergent species (monocots to dicots). Research studies have shown equivalent promoter function in more closely related species within the same family or between families such as the Solanaceae and Brassicaceae. In a preferred application, the promoter isolated from the anther-specific L10 gene discussed above is the promoter employed to regulate expression of the anti-sense gene.

In a further preferred application, the anther-specific TA39 promoter from tobacco is employed to regulate expression of the anti-sense gene. The anther-specific TA39 promoter is from a genomic clone from a commercially available genomic library from *Nicotiana tabacum* L. var NK 326 (Clontech). The library was probed with the anther-specific cDNA TA39 obtained from Dr. Robert B. Goldberg of UCLA. The isolation of TA39 and its expression characteristics have been reported (Koltunow, A. M., Truettner, J., Cox, K. H., Wallroth, M. and Goldberg R. B. Different temporal and spatial gene expression patterns occur during anther development. Plant Cell. 2:1201-1224, and Goldberg, R. B., Beals, T.

P., and Sanders, P. M. 1993. Anther Development: Basic Principles and Practical Applications. Plant Cell 5: 1217-1229 (1990).

5 It is appreciated, however, that refinements in promoter function may be required for individual plants or species to maximize or modulate the appropriate timing or level of expression to carry out aspects of the invention. Accordingly, methods for the modification of promoters to modify or improve function in various plants
10 of different origin are provided herein.

It is noted that there is not a high degree of DNA sequence homology between the native promoter essential for pollen development and/or function from the L4, L10, L16 and L19 clones. Test data reveal that the timing and
15 level of expression of these genes is not identical in pollen, but that all overlap in activity at some time.

It is expected that one may use any number of different promoters essential for pollen development and/or function to regulate the expression of a DNA
20 sequence in a recombinant DNA molecule of the invention. In order to determine what promoter essential for pollen development and/or function may be used to regulate the expression of an anti-sense gene (or other sequences which will be discussed below in regard to other
25 embodiments of the invention) consideration is to be given to a number of factors.

The promoter essential for pollen development and/or function used should be a promoter (or a modified form of the promoter) that is active at the appropriate time to
30 produce sufficient levels of transcribed RNA to carry out the invention. The promoter essential for pollen development and/or function derived from pollen specific clones disclosed herein (namely those derived from the microspore genes identified as L4, L10, L16 and L19) that
35 are active early in the development of microspores such that gene expression takes place both during and after the meiotic and mitotic division of pollen mother cells.

Thus, the activity of these promoters is not limited by segregation.

When using a promoter essential for pollen development and/or function to inactivate a gene that is essential to pollen formation and/or function, as hereinbefore discussed, it may be difficult to predict, a priori, which promoter essential for pollen development and/or function or modified promoter construct will effectively block the function of such a gene. It is preferable to use a promoter essential for pollen development and/or function that displays a similar developmental pattern to the gene. A convenient method to determine when the gene targeted for interference is expressed is to isolate RNA from developing microspores at different stages and to analyze this RNA for the expression of the gene by the so-called Northern blot analysis. This procedure will allow for the determination of the developmental period in which the gene is expressed. In order to determine the developmental period in which a promoter essential for pollen development and/or function gene is expressed, a similar series of analyses can be carried out using as a probe a reporter gene such as beta-glucuronidase joined to the promoter essential for pollen development and/or function, or a native gene from the same plant species from which a promoter essential for pollen development and/or function is isolated and whose expression is regulated by the promoter essential for pollen development and/or function. When the promoter essential for pollen development and/or function is isolated from one plant and used in a different plant species the preferred method is the use of a reporter gene joined to the promoter to determine the exact developmental timing that the promoter has in that particular plant species.

It is understood that the activity of a promoter essential for pollen development and/or function, whether intended for the same or different species may be modified in structure to change or alter activity in a

plant. Changes that are contemplated include but are not necessarily limited to: addition or deletion of sequences, orientation of upstream or downstream sequences, and the inclusion of introns or parts of the coding sequence of the pollen specific gene. The above
5 modification may serve to increase expression or improve regulation of expression to targeted stages of development.

It should be noted that the identification of a
10 promoter region (including constitutive, inducible and promoter essential for pollen development and/or function) is usually defined by function rather than a set DNA sequence. Two hundred (200) nucleotide bases or less of a promoter sequence may be sufficient to maintain
15 promoter function. It should also be recognized that some upstream DNA sequences can be arranged in opposite orientations and still retain or demonstrate enhanced promoter function. In addition, "enhancer-like" DNA sequences, which are usually small conserved DNA
20 sequences ranging in size from less than 10 nucleotides to considerably larger numbers of nucleotides may also be inserted into promoter regions to enhance expression.

It may also be desirable to include some intron sequences in the promoter constructs since the inclusion
25 of intron sequences in the coding region may result in enhanced expression. Thus, it may be advantageous to join the DNA sequences to be expressed to a promoter sequence that contains the first intron and exon sequences of a polypeptide which is unique to
30 cells/tissues of a plant essential for pollen formation and/or function.

Additionally regions of one promoter may be joined to regions from a different promoter in order to obtain the desired promoter activity. Specific examples of
35 chimeric promoter constructs are the chimeric promoters contained in the vectors PAL1107 and PAL1106.

It is desirable that the promoter essential for pollen development and/or function (or constitutive or

inducible promoter whichever is selected to construct a recombinant DNA molecule of the invention) function so that sufficient levels of anti-sense RNA are provided to substantially interfere with the expression of the targeted gene. Investigations of the mechanism of anti-sense RNA inhibition of gene expression in model systems have suggested that equal or greater than equal levels of anti-sense RNA may be required in order to observe a significant reduction of the targeted gene activity. However, in some cases it is noted that low levels of anti-sense RNA can have a specific reduction in sense gene activity. Therefore, in some instances if the gene that is targeted for inactivation by anti-sense RNA is a gene that is found to be essential for the continued development or function of all metabolically competent cells, or a gene that is expressed in all cell types at a low level, an excess of anti-sense RNA may not be required for inhibition. Additionally less than total reduction of the gene activity may be more than sufficient to disrupt pollen development which is known to be very sensitive to many stressful conditions. Therefore, it is suggested that the promoter essential for pollen development and/or function that is used, to carry out certain aspects of this invention, be chosen based on the observation that the promoter essential for pollen development and/or function functions to cause the expression of any sequences adjacent to it to be transcribed at a time that parallels or overlaps the period of time that the gene sought to be inactivated is expressed and that the levels of anti-sense RNA expressed from the anti-sense gene be of levels sufficient to inhibit the targeted gene's expression, usually to mean greater than or equal to the levels of sense RNA.

By using a promoter essential for pollen development and/or function to regulate the expression of an anti-sense gene, it is possible to interfere with for example, normal microspore development in any given plant, without having first to isolate from the genomic DNA of the plant

a gene which is essential to microspore development. Thus, a male sterile plant may be produced where the gene targeted for interference is a gene that is essential for the development and/or function of all metabolically competent cells/tissues. To produce a male sterile plant, such a gene is specifically interfered with by using a recombinant DNA molecule containing a promoter essential for pollen development and/or function to regulate the transcription of the anti-sense gene.

Furthermore, a male sterile plant may be produced by growing a plant which has a recombinant DNA molecule comprising an anti-sense gene to a gene which confers on cells of a plant resistance to a chemical agent or a naturally occurring or artificially induced physiological stress and a promoter essential for pollen development and/or function. The transcription product of the anti-sense gene will interfere with the expression of the targeted gene in cells/tissues essential to pollen formation and/or function. The gene confers on the remainder of the plant resistance to the stress.

In order to interfere with the expression of a gene, it is preferred that the anti-sense gene and targeted gene be expressed at about the same time. The coincident expression may be achieved in a variety of ways using combinations of constitutive, inducible and organ specific promoters (for example, a promoter essential for pollen development and/or function as hereinbefore described). However, co-incident expression may be readily achieved by regulating the expression of the anti-sense gene with the same promoter that controls the targeted gene, thereby causing both to be transcribed in the same time frame. The concept of regulating gene expression using anti-sense genes is described in Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews — Trends in Genetics, Vol. 1(1) 1986.

It is preferable that the recombinant DNA molecule containing an anti-sense gene of the invention further

contain one or more selection marker genes which encode a selection gene product which confers on cells/tissues of a plant resistance to a chemical agent or physiological stress, such that plant cells transformed with the recombinant DNA molecule or plants containing such transformed plant cells may be easily selected using a selective agent. Any effective selective agent for which a resistance gene has been identified may be used to select transformed plant cells. The selective agent used to select plant cells transformed with the recombinant DNA of the invention could be said to fall within two broad non-mutually exclusive categories, a chemical agent and a physiological stress. Examples of selective agents which may be used to select plant cells are herbicides, antibiotics, toxic substances and plant diseases, in particular glufosinate, glyphosate, tabtoxinine- β -lactam (TBL), chlorsulfuron, kanamycin, hygromycin, methotrexate, bleomycin, and phosphinotricin. Preferred selection marker genes which may be used in the present invention to facilitate selection of transformed cells include the gene encoding the enzyme hygromycin phosphotransferase conferring resistance to hygromycin and the enzyme neomycin phosphotransferase (NPT II) which confers resistance to kanamycin. The most preferred resistance markers include resistance genes to herbicide agents such as glufosinate (commercial names are Basta, Liberty) glyphosate (commercial name is Roundup) and TBL.

Examples of selective agents which may be used to select male sterile plants produced using a recombinant DNA molecule of the invention include a chemical agent, such as a herbicide, which could be used on a commercial scale and therefore can provide an economic benefit during the plant growing season additional and separate from the use of herbicide as a selective agent of a plant transformed by recombinant DNA. Resistance genes include any genes that allow the transformed cell to survive treatment with the selective agent. That includes, but is not limited to, a detoxification agent, interference

with transport of the selective agent, or over-production of the molecule targeted by the selective agent. Examples of herbicides for which a resistance gene has been identified which may be useful as selective agents for plants are glyphosate whose commercial name is Roundup (described in Comai, L., Facciotti, D., Hiatt, W.R., Thompson, G., Rose, R. E., Stalker, D. M., 1985, Nature, Vol. 317, Pages 741-744), chlorsulfuron (described in Haughn, G. W., and Somerville, C. R., 1986, Mol. Gen. Genet., Vol. 210, Pages 430-434) and phosphinotricin (Murakami T, et al., Mol. Gen. Genet. 205:42-50, 1986). Another herbicide for which a resistance gene or a fragment of DNA containing resistance gene(s) were reported is TBL (Kinscherf et al., 1991, J. Bact. 173: 4124-32, and from yeast, Marek, E.T. and Dickson, R.C., 1987, J. Bact. 169: 2440-8).

Genes encoding proteins or polypeptides that can provide a distinctive phenotypic characteristic to plant cells such as a gene that encodes a protein providing a distinguishable color or morphology to plant cells and genes which encode proteins which confer stress tolerance such as the metallothione gene may also be used as selection marker genes to facilitate the selection of transformed plants.

For a general reference on selective agents see Weising, K., Schell, J., and Kahl, G. Ann. Rev. of Genetics, 22:421-477 (1988).

The selection marker gene may be expressed in particular cells or constitutively in the entire plant depending on the nature of the selection gene product. The expression of the selection marker gene(s) is regulated by one or more selection marker promoters. The selection gene marker promoters may be isolated using techniques known in the art and include constitutive promoters such as CaMV 35S.

To facilitate selection of plant cells transformed with a recombinant DNA molecule or male sterile plants containing a recombinant DNA molecule it is preferred

that a selection marker gene be contained in the recombinant DNA molecule, most preferably linked to the DNA sequence contained in the recombinant DNA molecule. Where the recombinant DNA molecule contains more than one DNA sequence it is preferred that each DNA sequence be linked to a different selection marker gene. It is contemplated in some embodiments of the invention that at least two recombinant DNA molecules be used to produce a male sterile plant and in such embodiments it is preferable that each recombinant DNA molecule contain a selection marker gene preferably linked to the DNA sequence.

In a hybrid seed production scheme where there are alternating rows of male sterile plants and male fertile plants, it may be advantageous to carry out the final selection of male steriles in the field alongside the male fertile donors. Therefore it is desirable if the suitable male fertile donors are previously transformed to resistance to the selective agent to avoid having to selectively apply the selective agent to the rows of male sterile plants.

It is to be understood that a plant sought to be rendered male sterile need not be transformed with a selection marker gene that confers resistance to a chemical agent or a naturally occurring or artificially induced physiological or chemical stress if this gene is native to the plant.

The recombinant DNA molecule containing an anti-sense gene of the present invention preferably also contains a termination sequence and/or other transcription regulation signals. Examples of termination sequences which may be used in the recombinant DNA molecules of the invention are the nos terminator found in pRAJ-221 (Clonetech Laboratories, Palo, Alto, CA).

A detailed description of the isolation and identification of genes which are only expressed in cells/tissues of a plant essential to pollen formation

and/or function and promoters essential for pollen development and/or function is set out below:

5 To isolate genes which are only expressed in cells/tissues of a plant that are essential to pollen formation and/or function and promoters essential for pollen development and/or function, a genomic library of plant DNA may be constructed from DNA isolated from fresh young leaves according to standard methodology (Molecular Cloning, a Laboratory Manual Maniatis, T., Fritsch, E. 10 F., and Sambrooks, J., Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, 1982) and screened with probes derived from several tissues, one of which is made from RNA specific to cells/tissues of a plant that are essential to pollen formation and/or function 15 (hereinafter referred to as pollen specific RNA). The other probes should be made from RNA from different tissues so as to represent genes expressed in tissues of the plant that would not be expected to include genes that are expressed in cells/tissues of a plant that are essential to pollen formation and/or function. Examples 20 include but are not limited to such tissues as leaf, roots, seeds, stigma, stem and other organs. Some genes are expressed in all tissues and some only in a restricted number of tissues, by comparison of many plant tissues it is possible to isolate genes expressed 25 exclusively in cells/tissues of a plant that are essential to pollen formation and/or function.

Pollen specific RNA may be isolated from cells/tissues of a plant that are essential to pollen 30 formation and/or function that are at the early to late uninucleate stage. Although it is possible to isolate pollen specific RNA at other stages, isolation of the pollen specific RNA may be technically difficult at earlier developmental stages; older cells may have 35 limited nuclear gene activity; and, promoters may not be suitable for use as described in the present invention. For a detailed description of the isolation of pollen specific RNA see Example 1A herein.

Pollen specific RNA may be labelled for the purpose of detection. It is convenient to make radioactive cDNA by using the pollen specific RNA and AMV reverse transcriptase in the presence of random hexanucleotide primers and alpha-³²P]-dCTP. Probes are used for hybridization to nitrocellulose plaque lifts of plates containing the clones of the genomic library. Clones that can be identified as strongly hybridizing only to pollen specific cDNA and not cDNA from any other tissue examined are chosen. These clones are plaque purified and grown for DNA isolation. Alternative techniques for manipulation of DNA and RNA as well as recombinant DNA, growing and isolating clones can be found in standard laboratory manuals, such as Molecular Cloning, A Laboratory Manual (Maniatis, T., Fritsch, E. F., and Sambrook, J., Cold Spring Harbour Laboratory, New York, 1982).

For applications where the genomic DNA sequence of L4, L10, L16 or L19 from *Brassica napus* are used to carry out certain aspects of this invention, the preferred method to obtain a gene that is essential to pollen formation and/or function is to synthetically produce a homologous DNA sequence according to standard methodology (see Gait, M. J., Ed., {1984} *Oligonucleotide synthesis, a practical approach*, pp 1-22, IRL Press, Oxford, U.K.), label the sequence for the purpose of detection and use the labelled sequence to screen a *Brassica napus* genomic library produced according to the methods described.

The identity of the promoter and coding region of a given genomic clone is determined by restriction mapping and hybridization analysis. This may be accomplished by hybridization of cDNA probes made from pollen specific RNA with restriction fragments of the DNA clones immobilized on nitrocellulose. Restriction endonuclease fragments which contain both the coding region and regions of DNA on either side of the coding region are isolated by sub-cloning in appropriate vectors. Once isolated, it is convenient to use techniques such as S1

mapping and DNA sequencing to obtain exact coding regions and restriction sites within the sub-cloned DNA. This analysis is easily accomplished once the polarity with respect to gene transcription is known.

5 In order to determine the polarity of transcription of a gene, individual restriction fragments may be sub-cloned in commercially available vectors such as pGEM3, pGEM4, or pGEM3Z, pGEM4Z (available from Promega Biotech, Madison, Wisconsin, USA). By using these vectors one is
10 able to generate single stranded RNA probes which are complementary to one or the other strands of the DNA duplex in a given sub-clone. These strand specific probes are hybridized to mRNA, in order to establish the polarity of transcription. Among these probes, one can
15 isolate those probes which hybridize with and hence are complementary to the mRNA. Using this information it is possible to clearly determine from what DNA strand of the double-stranded genomic DNA molecule the mRNA has been transcribed.

20 In order to delineate and isolate the promoter DNA sequences of a given genomic clone the pGEM series of vectors can be used for the unidirectional deletion of sequences from the individual sub-clones in hybridization-protection experiments. Detailed
25 descriptions of these experimental procedures can be found in a number of laboratory handbooks and in the manufacturers technical notes supplied with the pGEM series of vectors. These experiments will clearly establish the promoter and coding regions of the pollen
30 specific genomic clones.

 The sequence of individual deletions in the pGEM vectors can be determined by dideoxy sequencing of plasmid mini-preps as described in the manufacturer's technical notes. Deletion sub-clones that are deleted to
35 very near the start of transcription or specific restriction fragments that encompass the promoter region or the promoter region and the start of transcription are chosen for the construction of genes that are expressed

only in developing microspores of pollen bearing plants. Usually the promoter fragment is inserted upstream of a terminator such as the nos terminator found in pRAJ-221 (available from Clontech Laboratories, Palo Alto, CA) and specific restriction fragments which are to be transcribed into anti-sense RNA are inserted between the promoter and terminator sequences. The entire construct is verified by combination of sequencing and restriction digests. The anti-sense gene thus constructed and verified may be inserted in T-DNA based vectors for plant cell transformation. T-DNA vectors that contain a selectable marker are preferred. It is to be understood that the anti-sense gene can be constructed in a variety of ways depending on the choice of vectors, restriction enzymes and individual genes used. For example, it may be convenient to insert restriction fragments intended to be transcribed into anti-sense RNA into a T-DNA based vector to which a promoter and terminator structure have been previously added. Alternatively, it is possible to insert a promoter fragment upstream of a coding region and terminator that has been previously added to a T-DNA based vector. In addition, it may be desirable in some crops not to insert the anti-sense gene into a T-DNA based vector but rather into a vector suitable for direct DNA uptake. Promoters other than promoters essential for pollen development and/or function can be used and joined with specific restriction fragments of genes and terminators provided that these promoters function in cells and/or tissues essential to pollen formation and/or function.

In accordance with a second embodiment of the invention, a method is provided for the production of a male sterile plant by transforming a plant with a recombinant DNA molecule comprising a promoter essential for pollen development and/or function as hereinbefore described, and a DNA sequence which encodes a gene product which renders a cell/tissue essential to pollen formation and/or function susceptible to a chemical agent

or physiological stress. Such a recombinant DNA molecule can be introduced into a plant wherein all the cells of the plant in which the recombinant DNA molecule is not expressed are resistant to the chemical agent and/or physiological stress.

In accordance with a third embodiment of the invention a method is provided for the production of a male sterile plant by transforming a plant with a recombinant DNA molecule comprising a promoter essential for pollen development and/or function as hereinbefore described and a DNA sequence which encodes a protein or polypeptide which is cytotoxic to a cell which is essential to pollen formation and/or function. By substance which is cytotoxic to a cell is meant a substance that when active disturbs the normal function of a plant cell/tissue preferably leading to cell death. Any cytotoxic substance which is known to be encoded by one or more identifiable DNA sequences may be employed within the scope of this embodiment of the invention, including but not limited to ricin, abrin and diphtheria toxin. Thus, the DNA sequence may encode any substance which is cytotoxic to a cell that is essential to pollen formation and/or function including the genes encoding ricin, abrin and diphtheria toxin.

In addition to DNA sequences encoding cellular toxins such as ricin, abrin and diphtheria toxin, the recombinant DNA molecule of the present invention may encode other cytotoxic substances including degrading or destructive enzymes such as ribonuclease, DNase, ribozymes, lipase, or protease, substances that disrupt or de-stabilize cytoplasmic integrity such as polylysine or poly-proline, substances that disrupt or de-stabilize proteins or polypeptides which are essential to certain biosynthetic pathways in plant cells or interfere with the expression of such proteins or polypeptides.

In accordance with a fourth embodiment of the invention, the recombinant DNA molecule may comprise one or more DNA sequences which encode a gene product which

renders a non-toxic substance cytotoxic to a cell/tissue which is essential to pollen formation and/or function. In particular, any identifiable DNA sequences encoding gene products which can convert a non-toxic to a cytotoxic substance can be employed within the scope of the invention. Examples of such DNA sequences include the following:

5 a) DNA sequences which encode indole acetamide hydrolase (IamH) which converts naphthalene acetamide to the plant growth regulator alpha naphthalene acetic acid (NAA) which is toxic to developing pollen grains, or converts indole acetamide to indole acetic acid (IAA) which is a plant growth regulator. One source of the enzyme IamH is the bacterium Agrobacterium tumefaciens (Inze, D., et al., 1984, Mol. Gen. Genet. 194:265-74).

10 b) DNA sequences which encode the enzyme methoxinine dehydrogenase (MDH) which converts non-toxic 2-amino-4-methoxy-butanoic acid (methoxinine) to toxic methoxyvinyl glycine. One source of MDH is the bacterium Pseudomonas aeruginosa (Margraff, R., et al., 1980, Experimentia 36: 486).

15 c) DNA sequences which encode the enzyme rhizobitoxine synthase which converts 2-amino-4-methoxy-butanoic acid to rhizobitoxine (2-amino-4-[2-amino-3-hydroxypropyl]-trans-3-butanoic acid). One source of the enzyme is the bacterium Rhizobium japonicum (Owens, L.D., et al., 1973, Weed Science 21: 63-66).

20 d) DNA sequences which encode the tissue specific beta-glucuronidase enzyme which for example converts a non-toxic analog of glucuronic acid conjugated to a toxic substance such as chloramphenicol (Gluc-Camp) or glyphosate (N-[phosphomethyl]glycine) to cytotoxic substances such as chloramphenicol and glyphosate, respectively. Other toxic substances may be conjugated to glucuronic acid to provide a non-toxic analog of glucuronic acid.

25 30 35 Where the non-toxic substance does not occur naturally in the plant cell into which the recombinant

DNA molecule of the fourth embodiment of the invention is to be introduced, the non-toxic substance may be introduced into the plant cell by spraying, watering or other similar means. The non-toxic substance may be produced in the cell by providing in the recombinant DNA molecule having a DNA sequence which encodes a gene product which renders the non-toxic substance cytotoxic to the cell, or in a second recombinant DNA molecule, a second DNA sequence which encodes the non-toxic substance. The non-toxic substance may also be introduced into the cell by providing in the recombinant DNA molecule having a DNA sequence which encodes a gene product which renders the non-toxic substance cytotoxic to the cell, or in a second recombinant DNA molecule, a second DNA sequence which encodes a gene product which converts a substance which is endogenous to the cell to the non-toxic substance. For example, a cell may contain a recombinant DNA molecule having a DNA sequence which encodes IamH (which converts indole acetamide to cytotoxic levels of indole acetic acid), and a recombinant DNA molecule having a DNA sequence which encodes IamS. IamS converts tryptophan which is generally endogenous to plant cells, to indole acetamide which in turn is converted by IamH to cytotoxic levels of indole acetic acid. In the examples above, where the introduction of more than one DNA sequence may be desired, the sequences can be introduced on separate recombinant DNA molecules or, in a preferred case, they can be linked together on one molecule.

The recombinant DNA molecule comprising one or more DNA sequences which encode a gene product which renders a non-toxic substance cytotoxic to a cell/tissue which is essential to pollen formation and/or function additionally contains one or more promoters to regulate the expression of the DNA sequences. If there is more than one DNA sequence in the recombinant DNA molecule the expression of the DNA sequences may be regulated by an identical promoter or the expression of each DNA sequence

may be regulated by a different promoter. Preferably, the expression of the DNA sequences is regulated by a promoter essential for pollen development and/or function as hereinbefore described so that the gene product which renders a non-toxic substance cytotoxic selectively interferes with the function and/or development of cells essential to pollen formation and/or function. Where the non-toxic substance is introduced into the plant cell by providing a second recombinant DNA molecule having a second DNA sequence encoding a second gene product which converts a substance which is endogenous to the cell to a non-toxic substance, the expression of the DNA sequence and the second DNA sequence may be regulated by either a promoter essential for pollen development and/or function, an inducible promoter or a constitutive promoter so long as there is selective interference with the function and/or development of cells essential to pollen formation and/or function. Preferably the expression of the DNA sequence encoding a gene product which renders a non-toxic substance cytotoxic is regulated by a promoter essential for pollen development and/or function and the expression of the second DNA sequence encoding a gene product which converts a substance which is endogenous to the cell to a non-toxic substance is regulated by a constitutive promoter, an inducible promoter or a promoter essential for pollen development and/or function, although most preferably by a promoter essential for pollen development and/or function. For a more detailed discussion of the above-mentioned promoters reference may be made to the discussion of promoters in reference to the recombinant DNA molecules containing anti-sense genes. A preferred promoter would be the microspore-specific Bp10 promoter (Albani, D., Sardana, R., Robert, L.S., Altosaar, I., Arnison, P.G., and Fabijanski, S.F., A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in

transgenic tobacco plants. *Plant J.* 2: 331-342 (1992) or the anther-specific TA39 promoter hereinbefore described.

5 In accordance with a fifth embodiment of the invention, the recombinant DNA molecule comprises one or more DNA sequences which encode a gene product which may be converted into a substance which is cytotoxic to cells/tissues essential for pollen formation and/or function.

10 The recombinant DNA molecule of the second, third, fourth and fifth embodiments of the invention also preferably further contain one or more selection marker genes as hereinbefore described and termination sequences and other transcription regulation signals as hereinbefore described. The preferred selection marker
15 would be herbicide resistance gene(s) to glufosinate or glyphosate or TBL, described hereinbefore.

The following description sets forth, in general terms, the steps that can be employed to produce plants which are male sterile or carry the male sterile trait,
20 using the recombinant DNA molecules of the invention. It is to be understood that these various steps may be accomplished by a variety of different procedures. In the following description of procedures, alternative ways to accomplish these steps are disclosed. Preferred
25 procedures may be detailed below and in the examples. However, it is contemplated that other variations will be apparent to those skilled in the art.

The recombinant DNA molecules of the invention may be used to produce a plant which is male sterile or
30 carries a male sterile trait, according to the following preferred steps:

- (1) Introducing one or more of the recombinant DNA molecules, as contemplated herein for carrying
35 out the invention, into the genome of one or more plant cells, of a plant selected from those species of pollen producing plants which are capable of being genetically transformed,

preferably cells from which differentiated whole plants may be conveniently recovered;

- (2) Selecting a plant cell into which the recombinant DNA molecule(s) is stably incorporated; and
- (3) Regenerating from the selected plant cell a plant which is male sterile or carries the male sterile trait.

A male sterile plant may be preferably produced by the above-mentioned steps by using the following recombinant DNA molecules of the invention in step (1):

- a) A recombinant DNA molecule comprising a DNA sequence which encodes an RNA which when produced in a cell of a plant which is essential to pollen formation and/or function interferes with a gene which is essential to pollen formation and/or function and a promoter essential for pollen development and/or function or a constitutive promoter;
- b) A recombinant DNA molecule comprising a DNA sequence which encodes an RNA which when produced in a cell of a plant which is essential to pollen formation and/or function interferes with a gene which is essential to the continued development and/or function of all metabolically competent cells of a plant and a promoter essential for pollen development and/or function; and
- c) A recombinant DNA molecule comprising a DNA sequence which encodes a protein or polypeptide which when produced in a cell of a plant which is essential to pollen formation and/or function is substantially cytotoxic to the cell or renders a non-toxic substance cytotoxic to the cells, where the non-toxic substance is introduced into the cell by converting a substance endogenous to the cell to the non-

toxic substance, and a promoter essential for pollen development and/or function.

A plant carrying a male sterile trait may be preferably produced by the above mentioned steps by using the following recombinant DNA molecules of the invention in step (1):

(a) A recombinant DNA molecule comprising a DNA sequence which encodes an RNA which when produced in a cell of a plant which is essential to pollen formation and/or function interferes with a gene which is essential to pollen formation and/or function and an inducible promoter;

(b) A recombinant DNA molecule comprising a DNA sequence which encodes a protein or polypeptide which renders a cell of a plant which is nominally resistant to a chemical agent or physiological stress sensitive to said chemical agent or physiological stress and a pollen-specific promoter; and

(c) A recombinant DNA molecule comprising a DNA sequence which encodes a protein or polypeptide which when produced in a cell of a plant which is essential to pollen formation and/or function renders a non-toxic substance substantially cytotoxic to the cell, where the nontoxic substance is externally applied to the cell, or where the non-toxic substance is introduced into the cell by converting a substance endogenous to the cell of the non-toxic substance and an inducible promoter controls the expression of the protein or polypeptide which converts the endogenous substance to the non-toxic substance.

A plant carrying a male sterile trait may be rendered male sterile by exposing the plant to a sterility actuating agent which for example is an inducer in the case of (a) above, a chemical agent or physiological

stress in the case of (b) above, or a non-toxic substance which is capable of being rendered cytotoxic (i.e., a cryptocytotoxic substance) to a cell which is essential to pollen formation and/or function in the case of (c) above.

A recombinant DNA molecule may be introduced into a plant cell by any one of a variety of known methods preferably by first inserting the recombinant DNA molecule into a suitable vector and then using the vector to introduce the recombinant DNA molecule into a plant cell. As hereinbefore described a recombinant DNA molecule may additionally contain a selection marker gene which encodes a selection gene product which confers on a plant cell resistance to a chemical agent or physiological stress, or confers a distinguishable phenotypic characteristic to the cells such that plant cells transformed with the recombinant DNA molecule may be easily selected using a selective agent. Transformed plant cells thus selected can be induced to differentiate into plant structures which will eventually yield whole plants. The selection agent can preferably be a herbicide such as glufosinate, glyphosate or TBL and the selection marker can be any gene conferring to the plant cell resistance to the herbicide. This approach has the added benefit that the resulting plant would also be herbicide resistant.

The use of Cauliflower Mosaic Virus (CaMV) (Howell, S. H., et al., 1980, Science 208: 1265) and gemini viruses (Goodman, R. M., 1981, J. Gen. Virol. 54: 9) as vectors has been suggested but by far the greatest reported successes have been with Agrobacteria sp. (Horsch, R. B., et al., 1985, Science 227: 1229-1231). Methods for the use of Agrobacterium based transformation systems have now been described for many different species. Generally strains of bacteria are used that harbour modified versions of the naturally occurring Ti plasmid such that DNA is transferred to the host plant without the subsequent formation of tumours. These

methods involve the insertion within the borders of the Ti plasmid the DNA to be inserted into the plant genome linked to a selection marker gene to facilitate selection of transformed cells. Bacteria and plant tissues are cultured together to allow transfer of foreign DNA into plant cells then transformed plants are regenerated on selection media. Any number of different organs and tissues can serve as targets for Agrobacterium mediated transformation as described specifically for members of the Brassicaceae. These include thin cell layers (Charest, P. J., et al., 1988, Theor. Appl. Genet. 75:438-444), hypocotyls (DeBlock, M., et al., 1989, Plant Physiol. 91:694-701), leaf discs (Feldman, K.A., and Marks, M. D., 1986, Plant Sci. 47:63-69), stems (Fry J., et al., Plant Cell Repts. 6:321-325 (1987), cotyledons (Moloney M.M., et al., Plant Cell Repts. 8:238-242 (1989) and embryoids (Neuhaus, G., et al., Theor. Appl. Genet. 75:30-36 (1987)). It is understood, however, that it may be desirable in some crops to choose a different tissue or method of transformation.

It is also understood that the successful transformation and recovery of a plant that contains these recombinant sequences may not always result in appropriate pollen specific expression. The transformation procedure results in the random insertion of foreign DNA such that "position effects" may override and suppress the activity of any introduced DNA. It is thus advisable to generate a number of individual transformed plants with any recombinant construct in order to recover individuals free from any limiting position effects. It may also be preferable to select plants that contain more than one copy of the introduced recombinant DNA molecule such that high levels of expression of the recombinant molecule are obtained.

It is known that the number of species of plants that have been successfully genetically transformed still represents a modest percentage of the total number of plant species that are of potential commercial interest.

It is true, however that the number of species that have been transformed has increased steadily and there is every reason to expect that transformation systems can be developed for any crop of interest in due course.

5 Routine transformation was initially achieved with species from two plant families: Solanaceae and Brassicaceae. Examples of species of commercial interest from within these families that have been transformed include: tobacco, Nicotiana tabacum L. tomato,
10 Lycopersicon esculentum Mill, potato, Solanum tuberosum L., and petunia, Petunia hybrida (Solanaceae); Canola/Rapeseed, Brassica napus L., cabbage, broccoli, kale etc., Brassica oleracea L., mustards, Brassica juncea L., Brassica nigra L., and Sinapis alba L.
15 (Brassicaceae).

Recently transformation has been reported of commercially important species from other families such as sugar beet, Beta vulgaris, (Chenopodiaceae), cucumber, Curcubita sp. (Curcubitaceae), cotton, Gossypium sp.,
20 (Malvaceae), sunflower, Helianthus annuus and lettuce Lactuca sativa, (Asteraceae=Compositae), and pea, Pisum sativum, soybean, Glycine max and alfalfa, Medicago sp (Fabaceae=Leguminosae). Transformation has also been achieved with tree species such as poplar, Populus sp.
25 (Salicaceae) and walnut, Juglans nigra, (Juglandaceae).

Transformation success with monocotyledonous species has not progressed as rapidly, since these species are generally not very susceptible to Agrobacterium mediated transformation. However, progress which has been
30 noteworthy includes asparagus, Asparagus officinalis; gladiolus, Gladiolus sp., (Lilaceae); corn, Zea mays and rice, Oryza sativa (Poaceae). The recent discovery that transformation with Agrobacterium can be accomplished by infecting germinating seeds without the requirement of
35 regeneration from cell culture (Chee, P.P., et al., *Plant Physiol.* 91:1212-1218 (1989) opens new horizons for species that may be difficult to regenerate. Additionally, widespread studies on the use of particle

guns to transfer microprojectiles coated in DNA into plant cells of species that are not readily susceptible to other methods holds great promise. It is expected that the present invention may be carried out with any
5 one of the above species and with any other species that is capable of being genetically transformed.

The most widely used and generally successful methods of introducing foreign DNA into plants are dependent upon the use of an infectious agent, such as the Agrobacterium tumefaciens Ti plasmid, as a vector for delivery of the
10 foreign DNA as hereinbefore described. However, it may be possible to use other methods, as well. Other methods that have been employed involve mechanical means such as direct DNA uptake, liposomes, electroporation (Guerche,
15 P. et al., 1987, Plant Science 52:111-16) and micro-injection (Neuhaus, G., et al., 1987, Theor. Appl. Genet. 75: 30-36). Recently the possibility of using microprojectiles and a gun or other devise to force small metal particles coated with DNA into cells has received
20 considerable attention (Klein, T.M. et al., 1987, Nature 327: 70-73). To date success with this and other mechanical methods has not been widely reported. The method of choice will be dependant on the particular plant species and will be apparent to those skilled in
25 the art. In those plant species where a successful transformation has not yet been demonstrated, it is anticipated that any new method of transformation developed may be used to insert the recombinant DNA molecules of the invention and the actual method of
30 insertion of the genes will have little or no effect on the functioning of the crop production systems described herein.

It may also be possible to produce plants which are male sterile or carry the male sterile trait by fusing
35 cells of a plant cell line containing cells having one or more recombinant DNA molecules of the invention with cells of plant species that cannot be transformed by standard methods. A fusion plant cell line is obtained

that carries a genetic component from both plant cells. Fused cells that carry the recombinant DNA molecule can be selected and in many cases regenerated into plants that are male sterile or carry the male sterile trait.

5 It is contemplated that some embodiments of the present invention may require that a plant cell be transformed with a recombinant DNA molecule containing at least two DNA sequences or be transformed with more than one recombinant DNA molecule. The DNA sequences or
10 recombinant DNA molecules in such embodiments may be physically linked, by being in the same vector, or physically separate on different vectors. A cell may be simultaneously transformed with more than one vector provided that each vector has a unique selection marker
15 gene. Alternatively, a cell may be transformed with more than one vector sequentially allowing an intermediate regeneration step after transformation with the first vector. Further, it may be possible to perform a sexual cross between individual plants or plant lines containing
20 different DNA sequence or recombinant DNA molecules. Preferably the DNA sequences or the recombinant molecules are linked or located on the same chromosome. It would then be possible to select from the progeny of the cross, plants containing both DNA sequences or recombinant DNA
25 molecules.

Where at least two recombinant DNA molecules are necessary to interfere with the function and/or development of a cell of a plant that is essential to pollen formation and/or function (e.g., a first
30 recombinant DNA molecule contains a first DNA sequence encoding a first gene product which converts a non-toxic substance to a cytotoxic substance and the non-toxic substance is introduced into the cell by means of a second recombinant DNA molecule which contains a second
35 DNA sequence which encodes a second gene product which converts a substance endogenous to a plant cell to the non-toxic substance), the recombinant DNA molecules may be linked to prevent segregation of DNA sequences

required to produce the desired effect. Alternatively, a male sterile plant can be produced by crossing two plant lines, each containing one of the recombinant DNA molecules, where the plant lines are otherwise isogenic.

5 Each plant line is preferably made homozygous, for the respective recombinant DNA molecules to ensure that all the progeny receive a copy of each of the recombinant DNA molecules. Further, in some hybridization schemes discussed below, it may be preferable to have the

10 respective recombinant DNA molecules located in the same chromosome pair in each line. The choice of a chromosome pair containing a first recombinant DNA molecule in one plant line may be predetermined, randomly, and the other plant line may be adjusted so that a second recombinant

15 DNA molecule is located on the same chromosome pair. For example, to produce a plant line which is homozygous for a first recombinant DNA molecule, a chromosome pair, in which a second recombinant DNA molecule is located is identified in a plant line, a transformed cell in which

20 the first recombinant DNA molecule is incorporated on the identified chromosome pair is then selected, a plant is regenerated from the transformed cell, the plant is selfed, and a plant is selected which is homozygous for the trait encoded by the first recombinant DNA molecule,

25 and the number of homozygous plants are increased, by selfing in isolation.

Methods of identifying the chromosome pair in which a recombinant DNA molecule is located and methods for producing homozygotes are discussed in detail below.

30 As indicated above, it may be desirable to produce plant lines which are homozygous for a particular gene. In some species this is accomplished rather easily by the use of anther culture or isolated microspore culture. This is especially true for the oil seed crop *Brassica napus* (Keller and Armstrong, Z. Pflanzenzucht 80: 100-108, 1978). By using these techniques, it is possible to

35 produce a haploid line that carries the inserted gene and then to double the chromosome number either spontaneously

or by the use of colchicine. This gives rise to a plant that is homozygous for the inserted gene, which can be easily assayed for if the inserted gene carries with it a suitable selection marker gene for detection of plants carrying that gene. Alternatively, plants may be self-fertilized, leading to the production of a mixture of seed that consists of, in the simplest case, three types, homozygous (25%), heterozygous (50%) and null (25%) for the inserted gene. Although it is relatively easy to score null plants from those that contain the gene, it is possible in practice to score the homozygous from heterozygous plants by southern blot analysis in which careful attention is paid to the loading of exactly equivalent amounts of DNA from the mixed population, and scoring heterozygotes by the intensity of the signal from a probe specific for the inserted gene. It is advisable to verify the results of the southern blot analysis by allowing each independent transformant to self-fertilize, since additional evidence for homozygosity can be obtained by the simple fact that if the plant was homozygous for the inserted gene, all of the subsequent plants from the selfed seed will contain the gene, while if the plant was heterozygous for the gene, the generation grown from the selfed seed will contain null plants. Therefore, with simple selfing one can easily select homozygous plant lines that can also be confirmed by southern blot analysis.

Two techniques may be used to produce plant lines which carry genes that segregate in a similar fashion or are on the same chromosome or a set of chromosome pairs. One may be a simple crossing strategy in which two transformants that are homozygous for a single inserted gene are crossed to produce F1 seed. The progeny plants from the F1 seed (F1 plant generation) may be crossed with a recipient plant and the segregation of the two inserted genes is determined (F2 plant generation). For example, where the IamH and IamS genes are the inserted genes, the F1 plants grown from the F1 seed will be male

sterile. If the original transformants are homozygous for a single inserted gene, when crossed with a non-transformed plant to produce F2 seed, the F2 plants will be 100% male fertile if the two transformants originally used for the production of the F1 seed carried the IamH and the IamS genes on the same chromosome or in the same linkage group. If the genes are in separate linkage groups or on different chromosomes, a variable degree of male sterility will be seen, in theory 25% of the plants will be male sterile if the genes segregate completely independently of each other. This approach allows for the selection of breeding lines from the homozygous transformed plant lines that contain the IamS and IamH genes which will segregate substantially 100% in the hybrid seed sold for commercial use.

An alternative strategy may make use of extensive genetic maps available for many commercially grown crops and the many easily scoreable markers that are known for most linkage groups or chromosomes. In some cases, linkage groups and chromosomes may be equivalent, whereas in others, there may be more than one linkage group assigned to each chromosome. When there is a marker for each chromosome, identification of the chromosome into which the recombinant gene has been inserted is relatively simple. A cross is made between each individual transformant and a recipient plant that allows for visualization of the marker(s).

If there are scoreable markers that have been localized to each of the chromosomes in the plant, and the markers are scoreable in the generation produced by this cross, one can localize the segregation of the inserted gene with the marker, thereby establishing the chromosomal location of that gene. This therefore allows for the chromosomal or more importantly the linkage group with which the inserted gene segregates. Many crops such as corn, tomato and many cereal crops have extensive genetic maps that allow for the identification of the chromosome containing the inserted gene. It is

contemplated that as more detailed chromosome maps are made, especially with the use of RFLP (restriction fragment length polymorphism) maps, the assignment of inserted genes to particular chromosomes will easily be
5 done for most commercial crop species.

As a means of confirmation, or in plant species where chromosomal markers are not known, it is possible to use a technique called pulse-field electrophoresis (originally described by Schwartz and Cantor, Cell, 37:
10 p67; 1984) to determine if different transformed plants contain inserted genes on the same chromosome. Pulse-field electrophoresis is a technique that can separate large DNA pieces, even chromosomal size, into a reproducible pattern on a gel. When this is done, it is
15 possible to process this gel such that the chromosome spots can be analyzed by southern blotting techniques, localizing the inserted gene to a chromosome spot. When the entire population of primary transformants are analyzed in this fashion, it is a simple task to choose
20 the two transformants that carry the inserted genes on the same chromosome spot.

As hereinbefore mentioned, after the recombinant DNA molecule(s) is introduced into the genome of a plant cell, a plant cell having a recombinant DNA molecule stably integrated in its genome is selected. This
25 selection step may be facilitated by incorporating a selection marker gene in the recombinant DNA molecule such that plant cells transformed with the recombinant DNA molecule may be easily selected using a selective agent as hereinbefore described. A selection marker gene is neomycin phosphotransferase (NPT II) which confers
30 resistance to kanamycin and the antibiotic G-418. Cells transformed with this selection marker gene may be selected for by testing in vitro phosphorylation of
35 kanamycin using techniques described in the literature or by testing for the presence of the mRNA coding for the NPT II gene by Northern blot analysis in RNA from the tissue of the transformed plant. A preferred selective

agent and selective marker would be a herbicide and its herbicide resistance gene(s) as described hereinbefore.

Expression of the recombinant DNA molecules of the invention in transformed plant cells may be monitored using Northern blot techniques. Single stranded RNA probes which are homologous to transcripts of the coding sequences of a recombinant DNA molecule(s) may be used to detect for the presence of the recombinant DNA molecule in a plant cell or tissue so that the expression of the coding sequence can be ascertained. It is preferable to use agarose gel electrophoresis to separate transcripts according to size under denaturing conditions. In the case where pollen specific gene expression of the coding sequence is sought to be accomplished it is advisable to test for the expression of the coding sequence in the cell type whose function and/or development is to be interfered with and in other tissues, such as leaves, roots, etc., so that tissue specific gene expression of the recombinant DNA molecule in such cell types can be verified.

The presence of a stably integrated recombinant DNA molecule in the genome of the plant cell may also be ascertained by using Southern blot techniques. In this procedure, total cellular or nuclear DNA is isolated from the transformed plant or plant cell and preferably digested with a restriction enzyme, thereby giving rise to discrete fragments. These discrete fragments may be detected in the nuclear or total DNA of the transformed plant or plant cells by employing standard gel electrophoresis, followed by nucleic acid hybridization techniques.

The formation of microspores in plants which contain the recombinant DNA molecule(s) of the invention and which are male sterile, is first monitored by visual microscopic examination of the anther structure. As maturation of the flower occurs, anther formation is expected to be delayed or completely inhibited such that no mature pollen grains are formed or released.

It will be appreciated that plants produced using the method for producing male sterile plants of the present invention may display varying degrees of male sterility. This may be a result of the nature of the plant cell transformed or of the chance positioning of the recombinant DNA molecule in the genome of the plant cell.

The following description of the invention sets forth, in general terms, the steps that can be employed to increase the number of male sterile plants and plants carrying the male sterile trait and to produce restorer plants, hybrid seed, hybrid seed with restored fertility and hybrid seed yielding a mixture of male fertile and male sterile plants. It is to be understood that these various steps may be accomplished by a variety of different procedures. In the following description of procedures, alternative ways to accomplish these steps are disclosed. Preferred procedures may be detailed below and in the examples. However, it is contemplated that other variations will be apparent to those skilled in the art.

As hereinbefore mentioned, the invention relates to hybrid seed having a genome comprising one or more of the recombinant DNA molecule(s) of the invention for producing plants which are male sterile or carry the male sterile trait and to seed of plants grown from the hybrid seed. The invention is also directed to a mixture of fertile and sterile hybrid seed and to seed of plants grown from said hybrid seed. The invention is also directed to hybrid seed having a genome comprising one or more of the recombinant DNA molecules of the invention for producing a male sterile plant and a corresponding restorer gene product for restoring fertility in the seed and to seed of plants grown from the hybrid seed.

To produce hybrid seed on a commercial scale from a plant which is male sterile or carries the male sterile trait, the number of such plants must be increased or maintained and crossed with a suitable male fertile parent line.

A plant which carries the male sterile trait may be more readily maintained because such plant will self-pollinate unless treated with a sterility actuating agent which renders the plant substantially sterile. For example, in a plant containing a recombinant DNA molecule having a DNA sequence which encodes a gene product that renders a cell susceptible to a chemical agent or physiological stress and having a promoter essential for pollen development and/or function, the plant will be rendered substantially male sterile by treating the plant with an actuating agent, namely the chemical agent or physiological stress. Accordingly, plants which carry the male sterile plant are preferably maintained by selfing, selecting from the selfing progeny a plant which is homozygous for the male sterile trait, and increasing the number of plants which are homozygous for the male sterile trait by selfing in isolation over a number of generations. Selection for a plant which is homozygous for the male sterile trait may also be carried out by the procedures discussed above.

A plant which is substantially male sterile is preferably maintained by crossing the male sterile plant with a suitable male fertile plant, obtaining seed from plants resulting from the cross, growing plants from the seed and selecting the plants which are male sterile from among the plants grown from the seed. This process may be repeated over a number of generations until the desired number of male sterile plants is obtained.

A plant which carries the male sterile trait may also be maintained by the latter method.

To facilitate selection of male sterile plants in the "maintained" plants, the recombinant DNA molecule(s) used to produce the male sterile plant should preferably comprise a selection marker gene and preferably the section marker gene is linked to a DNA sequence(s) contained in the recombinant DNA molecule as hereinbefore described. Such selection marker could be a herbicide resistance gene.

A suitable male fertile plant for the purpose of crossing with a male sterile plant to increase the number of male sterile may be, but is not limited to, a plant of the same inbred line from which the male sterile plant is derived. In some instances referred to below, maintenance of the male sterile line can be produced simply by selfing in isolation.

According to another scheme the number of male sterile plants may be increased by clonal propagation using tissue explants thereof, or other in vitro propagation techniques.

Where cost is warranted, and maintenance cannot be readily accomplished as discussed above, transformed plant cells may be grown in culture according to routine methodology to produce a cell line. A cell line may be regenerated according to routine methodology to increase and maintain the male sterile cell line. Routine methods for culturing cell lines and regenerating transformed plants from cell lines is described in standard plant tissue culture hand books. (Plant Tissue and Cell Culture, Green, C. E., Somers, D. A., Hackett W. P., and Biesboer, D. D. Eds, 1987, Alan R. Liss, Inc., New York, Experiments in Plant Tissue Culture, Dodds, J. H. and Roberts, L. W. Eds, 1985, Cambridge University Press, or Cell Structure and Somatic Cell Genetics of Plants, Vasil, I. K., Scowcroft, W.R., and Frey K.J., Eds., 1984, Academic Press, New York, Handbook of Plant Cell Culture, Vol 1-4, Evans, D. A., Sharp, W. R., Ammirato, P. V., and Yamada, Y. Eds., 1984-1986, Macmillan, New York, Bio-technology in Agriculture and Forestry, Vol 1 and 2, Bajaj, Y. P. S. Ed., 1986, Springer-Verlag, Berlin, or Plant Propagation by Tissue Culture - Handbook and Directory of Commercial Laboratories, George, E. F., and Sherrington, P. D., 1984, Eastern Press, Reading).

Production of hybrid seed may be accomplished by pollination of male sterile plants with pollen derived from selected male fertile plants. Pollination can be by any means, including but not limited to hand, wind or

insect pollination, or mechanical contact between the male fertile and male sterile plant. For production of hybrid seeds on a commercial scale in most plant species pollination by wind or by insects are preferred.

5 Selection of plants for pollen donation is determined by standard crossing of different plants with subsequent analysis of the progeny and selection of lines with the best combining ability and superior agronomic traits. Restoration of fertility in the hybrids may be

10 accomplished by using the methodology detailed below.

For certain crops of interest, such as vegetables, it may only be the leaves, stems or roots of the plant that are sold commercially. Therefore, even though the recombinant DNA molecule(s) which renders the plant male

15 sterile may be inherited and expressed in the hybrid plant it is not necessary to overcome or restore male fertility in the seed of the hybrid plant. However, for other crops, the commodity of commerce may be the seed or fruit produced by the hybrid plant. Thus for optimal

20 commercial utility of the hybrid it may be desirable to produce hybrid seed that is fertile.

The invention contemplates a variety of recombinant DNA molecules that may be used to produce a male sterile plant. In a scheme where any such recombinant DNA

25 molecule(s) consists of a single gene or linked genes, the gene(s) may segregate as a unit to produce a mixture of sterile hybrid seed and fertile hybrid seed. Similarly unlinked recombinant molecules may segregate to produce a mixture of fertile and sterile seed except, as

30 discussed below, where the molecules are located on different chromosomes of the same chromosome pair. In outcrossing species, the plants which grow from the fertile seed present may allow full pollination of the male sterile hybrid plants. Therefore, it may be

35 preferable to adopt a scheme to produce hybrid seed which is fully fertile. However in species which are relatively weakly outcrossing, male sterile hybrid plants will not be fully pollinated, thereby reducing the seed

yield. Therefore, where the commodity of commerce is the seed produced by the hybrid plant, it may be desirable to produce hybrid seed which is fully fertile.

5 The invention contemplates methods for restoring fertility in hybrid plants produced in accordance with the methods of the invention. Specifically, the invention contemplates a method for restoring hybrid plants produced in accordance with the methods of the invention by incorporating into a male parent plant, a restorer recombinant DNA molecule containing a restorer gene which encodes a restorer gene product which compensates for a gene function that has been compromised by a gene product(s) encoded by a first recombinant DNA molecule(s) of the invention, or which negates the effect caused by a gene product(s) encoded by a first recombinant DNA molecule(s) of the invention, as discussed below. The male parent plant thus may act as a "restorer plant". The restorer plant may be made homozygous for the gene encoding the restorer trait to ensure that all progeny inherit a gene. The homozygous restorer plant may be "maintained" by selfing in isolation to produce a "restorer line". Selection for a plant that is homozygous for the restorer trait may be carried out, as described above, by conducting anther or isolated microspore culture of the genetically transformed plant carrying the restorer trait, or preferably by selfing the plant in isolation prior to selection.

30 The expression of a restorer gene may be regulated by any promoter that is active during the period of transcription of a first recombinant DNA molecule which encodes a gene product which substantially interferes with the function and/or development of a cell essential to pollen formation and/or function. It is preferred that the expression of the restorer gene be regulated by the same promoter used to regulate the expression of the first recombinant DNA molecule or any promoter that is

highly active in tissues that are essential to pollen formation and/or function.

Methods for restoring fertility in hybrid plants which have integrated in the genome of their cells recombinant DNA molecules of the invention are discussed below.

The action of a protein or polypeptide which is substantially cytotoxic to a cell of a plant which is essential to pollen formation and/or function, and which is encoded by a DNA sequence contained in a recombinant DNA molecule which is integrated in the genome of cells of a hybrid plant may be negated by regulating the expression of the DNA sequence encoding the protein or polypeptide. For example, an anti-sense gene to the gene encoding the protein or polypeptide may be incorporated into a male parent line.

In particular where the recombinant DNA molecule has a DNA sequence which encodes a protein or polypeptide which is substantially cytotoxic to a cell of a plant which is essential to pollen formation and/or function, the male sterile plant may be crossed with a suitable male fertile plant that has been previously transformed with a restorer recombinant DNA molecule. The restorer recombinant DNA molecule may contain a restorer DNA sequence which is in the anti-sense orientation to that of the DNA sequence encoding the cytotoxic protein or polypeptide and a promoter controlling the restorer DNA sequence which activates transcription of the restorer DNA sequence at about the time of transcription of the DNA sequence. The restorer gene product inhibits the expression of the DNA sequence encoding the protein or polypeptide in the hybrid plant. The promoter controlling the expression of the restorer DNA sequence is preferably the same promoter essential for pollen development and/or function that controls the expression of the cytotoxic protein or polypeptides. Inducible and constitutive promoters may also be advantageously used to control the expression of the restorer DNA sequence.

A cytotoxic method of producing male sterile plants may involve the synthesis of proteins or polypeptides capable of substantially interfering with the function and/or development of a cell/tissue which is essential to pollen formation and/or function. Methods for restoring fertility in male sterile plants produced by cytotoxic methods involve the synthesis, in cells/tissues that are essential to pollen formation or function, of restorer gene products that specifically block, neutralize or destroy the proteins or polypeptides. If the recombinant DNA molecule integrated in the genome of a hybrid plant has a DNA sequence which encodes a cellular toxin, a restorer plant containing DNA sequences coding for a detoxifying molecule may be used to restore fertility. If the recombinant DNA molecule integrated in the genome of a hybrid plant has a DNA sequence which encodes a destructive enzyme, a restorer plant containing DNA sequences coding for a specific enzyme inhibitor may be used to restore fertility. If the recombinant DNA molecule integrated in the genome of a hybrid plant has a DNA sequence which encodes a cytoplasmic disrupting molecule, a restorer plant containing DNA sequences encoding a specific peptidase may be used to restore fertility. Judicious choice of the geneexpression regulation mechanism(s) for the male sterility producing agent would allow the use of gene expression mechanisms as restorer gene sequences. One example would be the use of the lexA mechanism (Brenn, R. and Ptashne, M. S., Regulation of Eukaryotic gene expression. Patent# 4,833,080. 1989). Specific examples of cytotoxic proteins or polypeptides and their restorer gene products are the proteinase enzyme trypsin and soybean or cowpea trypsin inhibitor; ribonuclease and a ribonuclease inhibitor; or a starch degrading enzyme such as alpha-amylase and an alpha-amylase inhibitor.

Fertility in a hybrid plant containing in the genome of its cells a recombinant DNA molecule having an anti-sense gene may be restored as follows. An anti-sense

gene contained in a recombinant DNA molecule to be incorporated in the genome of a plant may be selected such that it contains sequences of a sense gene which are transcribed, but not translated, in an anti-sense orientation. The transcribed but untranslated sequence may include an untranslated 5' leader sequence, intervening sequences and an untranslated 3' sequence, or any substantial fragments of these sequences. It is to be understood that the sequences or fragments thereof may be naturally occurring or foreign sequences.

Thus, any hybrid plant produced from a cross with a plant containing such an anti-sense gene may be restored by crossing with a restorer plant that has been transformed with a modified form of the initially targeted gene which does not contain the regions complementary to the anti-sense gene and therefore is not subject to the anti-sense regulation.

As described above, a plant carrying a male sterile trait may be produced by integrating various recombinant DNA molecules of the invention into the genome of a plant cell and regenerating the plant from the plant cell. The development and/or function of cells that are essential for pollen formation and/or function in the plant are interfered with only after the plant is exposed to a sterility actuating agent such as an inducer, a toxic agent or a cryptocytotoxic substance. Thus, restoration is inherent in growing plants produced from hybrid seed in the absence of the sterility actuating agent.

Where more than one recombinant DNA molecule of the invention is used to produce a male sterile plant, the recombinant DNA molecules may be inserted in the same chromosome pair in separate isogenic plant lines. The respective lines are preferably made homozygous for the respective recombinant DNA molecule(s)/gene prior to crossing the lines to produce a male sterile plant. Where a first and a second recombinant molecule are integrated into the same chromosome in the isogenic plant lines, a cross of these lines results in the first and

second recombinant DNA molecules being located on separate chromosomes of the same chromosome pair in the male sterile plant. Consequently, when the male sterile plant is crossed with a suitable male fertile plant of a different line, both chromosomes of the chromosome pair segregate into separate F1 progeny with the result that the first and second recombinant DNA molecules are not expressed in the same plant. Thus, the F1 hybrid seed is fully fertile. If the two recombinant DNA molecules are integrated into different chromosomes in the male sterile plant, then a portion of the F1 hybrid seed will be male sterile since there is a 25% probability of cosegregation of the chromosomes containing both recombinant DNA molecules into the male sterile plant. This latter approach may be advantageous with respect to outcrossing species. When the F1 male fertile plants outcross, a portion of the F2 seed will inherit both chromosomes containing the first and second recombinant DNA molecules and consequently will be male sterile. Where the seed is the commodity of commerce, it is advantageous for seed producing companies to use a scheme for hybrid seed production, where the saving of F1 hybrid seed is discouraged. The outcrossing in the F1 hybrid plants results in partial male sterility in the F2 generation, thereby reducing the seed yield of F2 plants, which is commercially desirable. An example of this method is as follows: a first male sterile plant line incorporating in its genome a recombinant DNA molecule having an IamH gene encoding IamH which converts nontoxic IAM to toxic levels of IAA, may be crossed with a second plant line having a genome incorporating a second recombinant DNA molecule having an IamS gene which converts tryptophan to IAM.

In a preferred method of the invention for producing hybrid seed a first male sterile plant line having a genome incorporating a recombinant DNA molecule having a first DNA sequence encoding a protein or polypeptide which renders a non-toxic substance substantially

cytotoxic to a cell of a plant which is essential to pollen formation and/or function and a promoter essential for pollen development and/or function is crossed with a second plant line which contains a second recombinant DNA molecule having a second DNA sequence which encodes a second gene product which converts a substance which is endogenous to a plant cell to the non-toxic substance. Preferably, the first and second plant lines used in this method are isogenic and each line carries a homozygous loci for the first DNA sequence or the second DNA sequence. Most preferably the first and second DNA sequences are located on the same chromosome pair of the plant lines, such that in any cross of the two lines a single chromosome pair contains both the first and second DNA sequences. The first plant line is made male sterile by exposing the first plant line to the non-toxic substance. The protein or polypeptide encoded by the recombinant DNA molecule incorporated in the genome of the first plant line will render the non-toxic substance toxic in cells of the plant which are essential to pollen formation and/or function, thus producing a male sterile plant line. The male sterile plant line also preferably has a selection marker gene linked to the first DNA sequence encoding the protein or polypeptide which renders a non-toxic substance cytotoxic to facilitate harvesting of the seeds having cells containing the first and second DNA sequences.

When the first male sterile plant line and the second plant line are crossed, the first male sterile plant line produces seeds having cells containing the DNA sequence encoding the non-toxic substance (e.g., IAM) and the DNA sequence encoding the protein or polypeptide (IamH) which renders the non-toxic substance cytotoxic (e.g., IamH converts IAM to toxic level of IAA). The seed having cells containing the first and second DNA sequences will produce male sterile plants which may be pollinated with a male fertile line to produce commercial hybrid seed. If the first and second DNA sequences are located on the

same chromosome or in the same linkage group, the DNA sequences will segregate completely in the F1 hybrid seed and the hybrid seed will be substantially male fertile.

5 Advantage is taken in the above-mentioned preferred method of the fact that most plant species produce, per plant, many hundreds of seeds. In oilseed Brassica for example, one plant, under normal conditions can produce one thousand seeds. Using the method described above, one can expect a thousand-fold increase in seeds per unit
10 area sprayed with the non-toxic substance. That is to say that, for example, when two isogenic lines are produced that carry the IamS and IamH genes, the first pre-production step involves the use of NAM to cause male sterility in the plant line that carries only the IamH
15 gene. When cross pollinated with the pollen from the plant that contains the IamS gene, one can expect up to one thousand seeds per unit area, each seed capable of growing into a male sterile plant. When these seeds are planted and crossed with a male fertile plant, one can
20 expect one thousand seeds per unit area. Therefore, if one were to plant one acre of the plant line carrying the IamH gene and the pollinator carrying the IamS gene, this acre would need to be sprayed with NAM. From this one acre however, enough seed would be obtained to grow 1000
25 acres of male sterile plants and pollinators, and from these 1000 acres, enough hybrid seed with restored fertility would be obtained to plant 1,000,000 acres of hybrid crop. The amount of management required to produce this hybrid seed is reduced over conventional
30 methods because of the pre-production amplification step employed. If the IamH gene is linked to a herbicide resistance gene, one can plant the fields randomly to ensure high rates of cross pollination and use the herbicide to kill the pollinator plants after flowering.
35 This method therefore allows for efficient hybrid seed production over methods where hybrid seed is harvested directly following the first cross pollination.

A particularly preferred embodiment of the above method is described in more detail below with reference to Figures 17 and 18. As illustrated in Figure 17, the method employs two plant lines which are homozygous, respectively, for the IamH gene (plant line A2) and the IamS (plant line A1) genes and otherwise isogenic. These genes are located on the same chromosome pair of the plant. Accordingly, plants produced from a cross of these two isogenic lines will contain the IamS and the IamH gene respectively on different chromosomes of a single chromosome pair. This will ensure that the two genes will segregate when this plant is crossed with a male fertile plant. To produce the hybrid seed, a two step procedure is used. The first step involves a pre-production of an isogenic male sterile line, the second step is the hybrid seed production itself. To accomplish the first step the following approach is used: The two isogenic lines A1 and A2 are planted in rows as shown, and when flowering starts, the plots are sprayed with NAM (naphthalene acetamide). This chemical is a non-toxic version of the plant growth regulator NAA, and the action of the IamH gene converts NAM to NAA. Under the control of the promoter essential for pollen development and/or function, the IamH gene only is expressed in pollen of the A2 line, and as such NAA is only made in pollen of the A2 line. Since NAA is a plant growth regulator, normal anther and microspore development is altered, leading to male sterility in the A2 plant line when treated with NAM. The plants which contain the IamS gene under the control of the promoter essential for pollen development and/or function (plant line A1) are not affected by the NAM, since they are unable to convert NAM to NAA, therefore these plants remain fully male fertile and can cross pollinate the A2 plants which have now become male sterile after treatment with NAM. On the A2 line, seed is produced that contains both the IamH and IamS genes under the control of promoters essential for pollen development and/or function (plant seed A2/A1).

The seed produced on the A2 line (plant seed A2/A1) is harvested. This harvesting can be done by harvesting specific rows. Alternatively, the IamH gene (A2) may be linked to a gene for herbicide resistance so that the herbicide can be used for the roguing of the plant line A1. Herbicide application takes place after flowering and will kill the A1 plants so that only seed that has the genotype A1/A2 is produced. The seed harvested from such a field will produce substantially 100% male sterile plants. The cross produces plants that express both the IamS and the IamH genes only in the pollen. This leads to the conversion of tryptophan, an amino acid normally found in plant cells to IAM (indole acetamide) via the IamS activity and finally to IAA (indole acetic acid) via the IamH activity. The molecule IAA is a plant growth regulator not normally found in substantial quantities in the developing pollen grains or anther of the plant. Since the IAA and the precursor Iam are small molecules that can be transferred from cell to cell via diffusion or active transport, altered growth regulator levels are seen throughout the anther. This altered growth regulator level leads to abnormality in pollen and anther development, producing a male sterile plant. This plant can be pollinated with a male fertile line leading to commercial hybrid seed. For the production of hybrid seed, the male sterile isogenic line can be planted in rows along side of a suitable male fertile plant, and the hybrid seed produced on the male sterile plant can be harvested. If the IamH gene is linked to a herbicide resistance gene, harvesting of hybrid seed is facilitated by using the herbicide to eliminate pollinator plants after cross pollination. The entire field can then be combined. All seed produced will therefore be hybrid. If the two genes (IamS and IamH) are located on the same chromosome or in the same linkage group, these two genes will segregate completely in the F1 hybrid seed. Since the plants will contain either the IamS or the IamH gene, but not both, the seed produced by this hybrid cross will

be substantially 100% male fertile. Therefore the plants grown from the seed of this cross will be fully fertile and set normal levels of seed. The F2 seed that results from the harvest of this field however will contain a
5 variable degree of male sterility, since in theory 12.5% or 2 out of 16 of the plants grown from this F2 seed will contain both the IamS and the IamH genes, as illustrated in Figure 7. Therefore, particularly in poorly
10 outcrossing species, the F2 generation will not be attractive to plant because up to 12.5% of the plants will not produce seed. Similarly, in outcrossing species which are not fully pollinated, a corresponding reduction in yield will be seen.

It is contemplated that as a variation of the above
15 particularly preferred method, a number of different ways of producing the toxic molecule specifically in pollen can be envisioned. In all approaches, at least one step in the production of the cytotoxic molecule has to take place specifically within the pollen cells or anthers.
20 For instance, it is possible to use a constitutively expressed IamS gene in a plant and to subsequently cross that plant with a plant that contains the IamH gene under the control of a promoter essential for pollen development and/or function such that Iam is produced in
25 all cells of the plant, but the growth regulator IAA is produced only in pollen cells due to the action of the pollen specific IamH gene. Conversely, it is possible to have IamH constitutively expressed in a plant, and cross this plant with a plant that contains a promoter
30 essential for pollen development and/or function driving the IamS gene. In this situation, the growth regulator IAA is only produced in pollen cells. It should be cautioned that in this case, one cannot use NAM to induce transitory male sterility in the plant that contains the
35 IamH gene, since that application of NAM would be lethal to the plant. In this case then hand pollination would be the preferred way of combining those genes. With regards to these methods the preferred embodiment of the

present invention places both the IamH gene and the IamS gene under the control of promoters essential for pollen development and/or function and preferably using the same promoter essential for pollen development and/or function or a promoter essential for pollen development and/or function whose expression substantially overlaps that of the other to each independently drive the expression of these two genes. Additionally, by linking the IamH gene to a selectable agent such as a herbicide, hybrid seed production is greatly facilitated. Any number of genes could be used to carry out this invention providing that the simultaneous production of two or more enzymatic or synthetic activities specifically in pollen leads to the production of a substance which is toxic or inhibitory to normal pollen growth or specifically interferes with anther or pollen development. This implies that one or more of these activities could be constitutive in the plant, but that the final combination of all enzyme activities be limited to pollen. It is also envisioned that one of these activities could be inducible by natural or artificial means such that sterility could be induced in plants. Specifically one embodiment of this method uses a plant line that carries a IamS gene under the control of an inducible promoter and a IamH gene under the control of a promoter essential for pollen development and/or function. These genes are preferably linked, but could be unlinked. When grown under inductive conditions, the plant becomes male sterile and can be pollinated by a suitable male fertile plant. The suitable plant could also carry a IamS gene under the control of a promoter essential for pollen development and/or function such that the progeny of this cross will be male sterile. These plants could then be crossed with a male fertile plant, producing hybrid seed. This seed would, depending on the location and number of the inserted genes, carry varying degrees of male sterility. It is also contemplated that a suitable male fertile plant be one that carries one or more copies of an anti-

sense gene that is capable of selectively inhibiting the expression of one or more of these genes such that 100% fertility is recovered in the plants grown from the hybrid seed.

5 Another particularly preferred embodiment of the invention uses IamS and IamH genes to produce a male sterile parent A as described herein, with the two genes introduced in Parent A as one recombinant DNA molecule, i.e., linked. This would have the advantage that the two
10 genes would not segregate in T1, as would be the case with the two genes being introduced into isogenic lines, on the same chromosomal pair. It is further proposed that the recombinant DNA molecule of this particularly preferred embodiment contains, linked to the IamS and
15 IamH genes, a gene that serves as a selection marker for the transformation. It is preferred that the selection marker be a herbicide resistance gene to glufosinate (commercial names are Basta and Liberty) or glyphosate (Roundup) or TBL as discussed hereinbefore. Parent A
20 would be maintained in a homozygous state for the gene(s) producing the male sterile trait (IamS and IamH in this embodiment) and for the resistance marker (herbicide resistance in this embodiment) by methods of gamete transformation as described hereinbefore. Parent A is
25 maintained by crossing with an isogenic, but not transformed, parent A and herbicide spraying, as shown in Figures 20 and 21.

Parent B would be the male parent. In a preferred case, it would be a plant line that was produced by
30 transformation with the herbicide resistance gene, using methods described hereinbefore and made homozygous for the herbicide resistance gene. As described in Figure 21, the hybrid seed would be 100% resistant to herbicide.

In an alternative embodiment, if a male fertile seed
35 is desired, parent B would be produced by using a recombinant DNA molecule containing both male fertility restoration and herbicide resistance genes as described

in Figure 20. The resulting hybrid seed is 100% herbicide resistant and 100% male fertile.

Of course, parent A could be produced by crossing isogenic lines, each containing one of the two genes, i.e., IamS and IamH, linked to the herbicide resistance marker. Both the plant carrying the IamS and the plant carrying the IamH genes are made homozygous for the genes by methods of transforming anther or microspore cell culture or by plant selfing as described herein. This is illustrated in Figure 22a. Parent A is selected and maintained as described above and as illustrated in Figure 22b. Parent A is crossed with parent B, produced as above. The hybrid seed is 100% herbicide resistant and, if parent B contains a male restoration gene(s), then the hybrid seed is 100% male fertile. The plant resulting from the cross and the hybrid seed is 100% herbicide resistant and either 100% male sterile or 100% male fertile, as determined by which recombinant DNA molecule was chosen to be introduced in the genome of parent B. DNA sequences that can serve to restore male fertility to a hybrid plant, where one parent was made male sterile were discussed hereinbefore. A preferred restorer gene would be an anti-sense gene to either gene 1 and/or gene 2 (IamS and IamH genes, described above). The use of anti-sense gene(s) was described herein. Another preferred male fertility system in the hybrid plant would utilize, gene expression repressor mechanism(s), especially the lexA system discussed hereinbefore. A transactivator can also be converted to a repressor to create a dominant negative inhibitor which could inhibit the expression of the "target" promoters linked to gene 1 and/or gene 2. In yet another preferred embodiment, judicious construction of the promoter sequences so as they contain recognition sites for binding by a transactivator or transdominator would allow greater control of IamS and/or IamH expression. If the transdominator sequences were themselves inducible by some externally applicable chemical, one could propagate

the plant with male sterile genes as a fertile plant until the need to utilize such plant as male sterile in a hybrid cross.

To produce a male sterile parent A for the hybrid by use of the linked gene 1 and gene 2 scheme described above, it would be essential that expression of at least gene 1, or gene 2, or both, be regulated by employing a promoter active only in cells involved in pollen formation/ structure. Such promoters were described hereinbefore. A preferred case would employ the anther-specific promoters described herein, Bp10 delivered from Brassica gene L10 and/or the anther-specific gene from tobacco, TA39. Herbicide resistance gene(s) could be constitutively expressed.

In Figure 1, a schematic representation of the production of the anti-sense vector PAL1302 is shown. A plasmid containing the GUS gene (Beta-glucuronidase, described in Jefferson, R.A., Plant Molecular Biology Reporter, 1987, 5: 387-405) in the anti-sense orientation flanked by the CaMV 35S promoter and the nos termination signal was obtained from the vector pBI221.1 (available from Clontech Laboratories, Palo Alto, CA, USA). The GUS coding sequence found between the CaMV 35S promoter and the nos ter of the vector pBI221.1 was excised and digested with the restriction enzymes SmaI and SstI. The SstI site was made blunt-ended using the Klenow fragment of DNA polymerase I and the blunt-ended vector and the GUS coding sequence were relegated. A plasmid (pPAL303) containing the Gus coding sequence inverted with respect to the direction of transcription of the CaMV 35S promoter was identified.

The binary vector PAL1302 containing the anti-sense GUS gene was constructed using the vector pVU1011 (obtained from The Plant Breeding Institute, Cambridge, UK). pVU1011 contains the hygromycin phosphotransferase coding sequence flanked by the CaMV 35S promoter and the nos ter inserted into the polylinker of the Agrobacterium binary vector Bin19 described by Bevan, M., Nucl. Acids

Res. 1984, 12: 8711-8721. The vector pVU1011 can confer both hygromycin and kanamycin resistance to transformed plant cells. The insertion of the CaMV35S promoter anti-sense GUS nos ter fragment into pVU1011 was accomplished in such a way as to inactivate the NPTII gene of this vector and was performed as follows. A small SphI - PstI restriction fragment containing the right border (RB), the NOS promoter and the beginning of the NPT II coding sequence of pVU1011 was first subcloned into the SphI and PstI sites preceding the CaMV 35S promoter of pPAL303 to form pPAL306. Digestion of pPAL306 with SphI and EcoRI released a fragment consisting of the RB, the NOS promoter, the beginning of the NPT II coding sequence and the CaMV 35S promoter - anti-sense GUS noster construct. This fragment was then ligated into the SphI sites of pVU1011 by adding to the ligation pGEM-4Z (Promega Biotech, Madison, WI, USA) cut with EcoRI and SphI to provide a small fragment of polylinker as a bridge between the SphI site of pVU1011 and the EcoRI site of the insert from pPAL306 respectively. The orientation of the insert was verified and a binary vector (PAL1302) possessing a reconstructed RB fragment and a NPT II gene inactivated by the CaMV 35S promoter - anti-sense GUS gene-noster insertion was identified. This vector can confer only hygromycin resistance to plants and carries the anti-sense GUS gene.

In Figure 2a-d the orientation of the genes contained within the four microspore specific clones from *Brassica napus* are from 5' to 3'. As shown, the 5' region corresponds to the promoter region and is identified with a small arrowhead. The 3' region delineates the end point of transcription of the gene. Clones L4, L10 and L19 were used for the isolation of microspore specific promoter fragments and for the isolation of microspore specific coding regions. The non-transcribed regions are identified as a single thin line, while the regions of the clones that are transcribed are demarcated by a boxed area. Within this boxed area the portion of the

transcribed DNA that represents the exon regions is demarcated by being filled in black while the intron sequences are left unfilled. The approximate regions of DNA sequenced for clones L10, L16 and L19 are shown by underlining. Restriction sites identified are those that are relevant to the constructs detailed below. The right and left arms of the lambda cloning vectors are not shown.

In Figures 3a-d, the complete DNA sequence of the clone L4 is shown along with the DNA sequence of the portions of the clones L10, L16 and L19 that are identified in Figures 2a-d. In Figure 3a, clone L4, nucleotide 1 in the complete sequence is at the left-most EcoRI site while nucleotide 8579 is at the first nucleotide of the right-most EcoRI site. The start of transcription of gene 1 in clone L4 is nucleotide 235. The 5' and 3' intron splice sites are identified in boldface type. The start ATG codon is shown as well as the stop termination codon. The deduced amino acid sequence of the proteins encoded for by these genes are also shown. The end of transcription for gene 1 is approximately nucleotide 1427. The second gene in clone L4 is most likely non-functional due to a insertion and a deletion that occurs in the region of the promoter and first exon. This gene was not utilized for constructs. The third gene in clone L4 has a transcriptional start at position number 6298 in the DNA sequence and transcription ends at approximately nucleotide 7490. The ATG start codon, intron splice sites and termination stop codon are all identified as above. Vectors were constructed from this clone by using promoter fragments from both genes 1 and 2, as well as promoter fragments from genes 1 and 2 that contained the first exon and intron sequences and a short portion of the second exon for each of the genes. The specific promoter fragment constructs are detailed below.

In Figure 3b, the nucleotide sequence of the region of clone L10 demarcated in Figure 2b is shown. The start

of transcription is at nucleotide 1. In this sequence the ATG start codon is at nucleotides 45-47, the first exon ends at nucleotide 315 the second exon starts at nucleotide 476 and extends to nucleotide 1586. The third exon starts at 1673 and extends approximately to nucleotide 1989. The precise end of transcription was not determined. The deduced amino acid sequence is also shown. For some promoter constructs the region of the clone 5' to the sequenced portion were used. The specific details of the constructs are listed below.

In Figure 3c, the nucleotide sequence of clone L15 is shown. Clone L16 shows considerable homology to clone L10 specifically in the portions of the two clones that code for protein sequence. The intron sequences between the two clones differ considerably however. Clone L16 does not contain a 5' promoter region and as such was used only as a source of coding sequences for anti-sense RNA constructs. Nucleotide 1 demarcates an EcoRI site that occurs in a coding region of the DNA that is homologous to the first exon of clone L10. By homology, this coding region extends to nucleotide 124, where the first intron is located. This intron, which is located at the same relative position as the first intron of clone 10, is longer than the intron in clone L10 and extends to nucleotide 688. Nucleotide 689 is the start of the second exon and this exon, which shows strong homology to the second exon of clone L10, extends to nucleotide 1793. There is a second intron at this point and this intron extends to nucleotide 1909. The third exon starts at 1910 and extends to approximately nucleotide 2210. The deduced amino acid sequence is also shown for specific regions of the clone that show considerable homology to clone L10. The precise nucleotide where transcription stops has not been determined.

In Figure 3d the nucleotide sequence of the region of clone L19 demarcated in Figure 2d is shown. The start of transcription is located at position 1 in the

sequence. The ATG start codon is at nucleotides 136-138 and the first intron starts at nucleotide 1201. This intron ends at nucleotide 1338 wherein the second exon starts. The end of transcription occurs at approximately nucleotide 2074. The deduced amino acid sequence is also shown.

In Figure 4, the DNA sequences of three cDNA clones that are homologous to the genes contained in the clone L4 are shown. The DNA sequence of these three cDNA clones as well as the sequence of the correctly spliced transcribed regions of genes Bp4A and Bp4C in the genomic clone L4 are aligned, only the nucleotide differences within these clones are shown, nucleotides that are conserved between the sequences are only shown on the upper sequence. The asterisks shown in Figure 4 mark the 5' end of the cDNA clones of cBp401, cBp405 and cBp408.

In Figure 5, the partial nucleotide sequence of a cDNA clone that is homologous to the coding region of clone L10 is shown. This cDNA clone is approximately 1.3 Kb in length and has EcoRI sites at the 5' and 3' ends of the cDNA sequence that were added via synthetic linkers in the cDNA cloning procedure.

In Figure 6, the nucleotide sequence of the cDNA clone that corresponds to the coding region of clone L19 is shown. Identified in this sequence is the EcoRV site present at the 5' end of the transcribed region of L19. A portion of the poly A tail is shown. Not shown are the EcoRI sites that were added as linkers in the cDNA cloning procedure; these sites are present adjacent to the 5' and 3' ends of the cDNA clone.

In Figure 7 (a,b,c,d,e) the construction of 6 vectors containing promoter and promoter fragments from the clone L4 is described. The first step in the construction of these vectors was accomplished by first subcloning the EcoRI-SstI(nucl.1-2132) fragment containing the first gene of clone L4 (235 base pairs of promoter/exon/intron/second exon) in the commercially available vector pGEM-4Z(Promega Biotech, Madison, WI,

USA) using the EcoRI-SstI sites of the polylinker of this vector. This plasmid was named pPAL0402. The 2.7Kb EcoRI fragment of clone L4 that contains the third gene (Bp4C) was then cloned into the EcoRI site of pGEM 4Z, leading to a plasmid called pPAL0411. The plasmid pPAL0402 was then digested with EcoRI and the 2.7 Kb EcoRI fragment from pPAL0411(nucl. 5859-8579) that contains the gene number three (Bp4C) from clone L4 was added to it. Clones were recovered that contained this inserted 2.7 Kb EcoRI fragment in both orientations relative to the promoter region of the first gene. A clone that contained this third gene fragment in a orientation such that the promoter from the third gene was opposite to the promoter in the first gene was chosen and called pPAL0403. The plasmid pPAL0403 contains the entire third gene from clone L4 oriented in such a fashion as to have the promoter region immediately adjacent to the 235 basepair promoter region of the first gene in pPAL0403. This plasmid, pPAL0403 was digested with DdeI, producing a fragment of approximately 1.9 Kb. The DdeI sites are located at nucleotides 303 and 7366. Because of the orientation of these fragments, digestion with DdeI produces a 1.9 Kb fragment. This 1.9 Kb fragment contains a copy of the third gene (Bp4C) oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 (Bp4A) which is transcribed from left to right, ending in a DdeI site that is located 67 base pairs downstream of the major start site of transcription and precedes that ATG start of translation codon by 2 nucleotides. This 1.9 Kb DdeI fragment was made blunt with Klenow fragment and cloned into the XbaI site of the polylinker region of pGEM 4Z previously made blunt ended with Klenow fragment. The resultant plasmid pPAL0408, was recovered and subsequently was digested with SalI and SstI, which releases the cloned DdeI fragment bordered by on the left hand side, (nucl 7366) SalI and on the right hand side

(nucl 303) of this construct and contains a portion of the polylinker of pGEM 4Z containing the following unique sites: BamHI, SmaI, KpnI, and SstI restriction enzyme sites. This SalI - SstI fragment was cloned into the SalI - SstI sites of PAL1001. PAL1001 is the binary vector Bin19 (described by Bevan, M., Nucleic Acids Res., 1984, 12:8711-8721) to which has been added the nos ter polyadenylation signal as a 260 bp SstI - EcoRI fragment isolated from the plasmid pRAJ221 (available from Clonetech Laboratories, Palo Alto, CA USA) in the SstI - EcoRI sites of the polylinker region of Bin19. This nos ter is identified as a stippled box. The binary transformation vector that resulted from the insertion of the SalI - SstI fragment of pPAL0408 into PAL1001 was named PAL1107. The details of the construction are shown in Figure 7a. This vector has a copy of the third gene oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 which is transcribed from left to right, followed by a polylinker with unique sites for the insertion of DNA which consist of: BamHI, SmaI, KpnI and SstI followed by the nos ter signal. This vector has the feature in that additional 5' non-coding sequences were placed upstream to the 235 base pair core promoter on Bp4A, but these additional 5' sequences were in a opposite orientation. The provision of these sequences in this orientation does not affect the pollen specificity of the core 235 base pair promoter.

In addition to this vector, similarly structured vectors were made which contained essentially the same type of gene promoter arrangement but contained the intron of the first gene (Bp4A) of clone L4. Intron sequences in plant genes have been shown in some cases to play a role in gene expression. This intron containing vector was constructed by making a deletion series of the clone pPAL0402. pPAL0402 was first digested with PstI and SmaI. Exonuclease III was used to unidirectionally

digest the DNA as shown (Figure 7b). After S1 nuclease treatment and repair with Klenow, the plasmid was relegated and clones that have had different portions of the coding regions of gene Bp4A digested out of them were recovered. Deletion subclones were sequenced. One was chosen for vector constructs. This is referred to as deletion 23B. This subclone represented a deletion that has most of the second exon of gene Bp4A removed but contains the intron splice site and first exon of gene Bp4A. This subclone contains a portion of the clone L4 that extends from nucleotide 1 to nucleotide 1166. To this subclone was added the 2.7 Kb EcoRI fragment from pPAL0411 that contains the third gene of L4 (Bp4C) in such an orientation that the direction of transcription of the third gene is from right to left (as in PAL1107, pPAL0408), fused to the 235 base pair promoter region from the first gene of clone L4 which is oriented to transcribe from left to right followed by the first exon of gene 1, the entire intron of gene 1 and 33 nucleotides of the second exon of gene Bp4A from clone L4. This plasmid containing deletion 23B and the 2.7 Kb EcoRI fragment containing the third gene fragment was named pPAL0406. This plasmid was digested with HindIII, which yields a fragment containing a small portion of the promoter of the third gene as well as the entire promoter of the first gene, first exon, intron and a portion of the second exon. This HindIII fragment was inserted into the HindIII site of PAL1001, resulting in the vector PAL1106 (deletion 23B derived). This vector has in the following order, A portion of the promoter from the third gene in clone L4, the entire 235 base pair promoter of the first gene in clone L4, followed by the first exon, the intron and a portion of the second exon of gene 1 of clone L4, followed by a polylinker containing the following unique cloning sites: SalI, XbaI, BamHI, SmaI, KpnI and SstI and the nos ter polyadenylation signal. The construct is shown in Figure 7b.

Additional constructs with the promoter regions of the genes contained in clone L4 were done in order to provide a number of suitable vectors that are useful for pollen specific expression of gene sequences. The three genes within clone L4 (Bp4A, Bp4B, Bp4C) show very near-exact DNA homology and this is most apparent between the first (Bp4A) and third (Bp4C) gene. The second gene (Bp4B) is a homologous copy that has undergone sequence changes that have appear to have lead to inactivation. The extensive similarity between the first, second and third genes in clone L4 is also maintained in the promoter region such that out of the first 235 nucleotides of the first and third gene promoter regions there are only 5 nucleotides that differ between them. Downstream of the TATA box in these two promoters the only difference between them is the presence of one additional nucleotide at the start of transcription. For example, comparison of Promoter 1, Bp4A, partially represented as:TATGTTTtAAAA with Promoter 3, Bp4C, partially represented as:TATGTTTAAAA. shows that the transcribed region underlined and the single nucleotide difference in lower case. However, within the sequence of the first gene there is a nucleotide change that introduces a DdeI site (nucl. 303) in the untranslated 5' leader sequence upstream of the ATG start codon that is not present in the untranscribed leader sequence of the third gene in clone L4. Chimeric promoter constructs were made which utilized this DdeI site in the first gene to combine with sequences from the third gene promoter. The region of the first promoter used for these constructs consisted of the sequences contained between the SnaBI site (nucl 21) near the TATA box to the DdeI site located immediately upstream of the ATG start codon in the first gene (nucleotide 303 is the first nucleotide in the recognition sequence for DdeI). The other region of this chimeric promoter (5' of the TATA box) was a fragment extending from the EcoRI site of the third promoter (nucleotide 5858) to the SnaBI site

near the TATA box (nucleotide 6273). Therefore to facilitate construction of these pollen specific vectors, the following reconstructions were performed.

5 The EcoRI to DdeI fragment that encompasses the promoter region of the first gene in clone L4 was isolated by first cutting pPAL0402 with DdeI, blunting with Klenow, and then cutting with EcoRI. The 235 base pair fragment corresponding to this region was cloned into the EcoRI - SmaI sites of pGEM 4Z. This plasmid 10 (pPAL0422), was then cut with EcoRI and SnaBI. A DNA fragment that contained the EcoRI to SnaBI portion of the promoter for gene 3 in clone L4 was isolated by digesting pPAL0411 with EcoRI and SnaBI. This released an approximately 415 base pair EcoRI (nucl. 5858) to SnaBI 15 (nucl. 6273) fragment that represents most of the 5' region of the gene 3 promoter from clone L4 (the SnaBI recognition site is 2 base pairs downstream of the TATA box). This EcoRI - SnaBI fragment was used to replace the shorter EcoRI - SnaBI fragment removed for the first 20 promoter subclone (pPAL0422), reconstructing a promoter fragment of approximately 550 base pairs. This plasmid is referred to as pPAL0421. This chimeric promoter fragment contains 415 base pairs of the promoter of gene three in clone L 4, followed by approximately 99 25 Nucleotides of the first gene promoter/untranslated leader sequence.

For construction of a pollen specific cassette vector, the following plasmids were first constructed. The first plasmid constructed contained the nos ter 30 polyadenylation signal with a polylinker in front of the nos ter signal. This was accomplished by first isolating from pRAJ221 the nos ter as a SstI - EcoRI fragment and this fragment was cloned in pGEM 4Z using the SstI and EcoRI sites in the polylinker. This subcloned is 35 referred to as pPAL001. To pPAL001, a fragment coding for neomycin phosphotransferase (NPT II) derived from the plasmid pRAJ162 was added to it in the anti-sense orientation as follows: The plasmid pRAJ162 contains the

NPT II gene from the transposon TN5 inserted as a SalI fragment and bounded by a polylinker in the plasmid pUC-9 (which was obtained from the Plant Breeding Institute, Cambridge, UK). pRAJ162 was digested with HindIII and SmaI. The DNA fragment containing the NPT II gene was isolated by elution from an agarose gel. pPAL001 was digested with HindIII and SmaI and the NPT II gene fragment was inserted. The resultant plasmid was called pPAL002 and had such orientation of restriction sites and the NPT II gene and nos ter as follows: HindIII, PstI, SalI, 3' end NPT II coding sequence 5' end, SalI, BamHI; SmaI, KpnI, SstI, nos ter, EcoRI. pPAL002 was cut with HindIII and the site made blunt ended by the use of Klenow fragment. pPAL0421 was digested with HincII and pVUII, both of which leave blunt ends, and the promoter fragment was ligated into HindIII cut blunt ended pPAL002. Plasmids were obtained that contained the promoter in both orientations relative to the nos ter signal. One plasmid was chosen with the proper orientation (5' promoter/anti-sense NPT II/nos ter) and was named pPAL0419. pPAL0419 has the following DNA fragments: A small (approx. 130 bp) of pGEM 4Z that contains the SP6 promoter, the 550 base pair chimeric promoter, the NPT II gene in the anti-sense orientation relative to the promoter, followed by the nos ter polyadenylation signal. This entire promoter/NPT II/nos ter construct is excisable by EcoRI. pPAL0419 was digested with EcoRI, and the promoter NPT II nos ter structure was cloned into Bin19 using the single EcoRI site in the polylinker of Bin19. The resultant transformation vector was named PAL1419. In addition to the anti-sense NPT II gene, the vector contains a constitutive NPT II gene under the control of the nos promoter. This vector therefore confers resistance to kanamycin in all cell types with the exception of pollen cells where the gene expression from the constitutive promoter is inhibited by the anti-sense RNA produced from

the promoter/NPT II/nos ter construct contained in PAL1419.

5 In order to provide promoter sequences that could be utilized with additional gene constructs, the plasmid pPAL0419 was digested with SalI. This digest removes the NPT II coding region and this SalI digested pPAL0419 was relegated giving rise to pPAL0420. pPAL0420 represents the promoter essential for pollen development and/or function followed by a polylinker for insertion of genes
10 that has the following unique sites: HincII, PstI, SalI, BamHI, SmaI, KpnI, SstI, followed by the nos ter polyadenylation signal. The entire promoter/polylinker/nos ter construct can be conveniently excised as a single EcoRI fragment. The details of this
15 construct is shown in Figure 7c.

For additional promoters essential for pollen development and/or function constructs, the following approach was used. The intact L4 clone in the lambda cloning vector was digested to completion with the
20 restriction enzymes SstI and HhaI. The resultant fragments were separated by gel electrophoresis and a 2.65 Kb fragment that contains the promoter/first exon/intron/partial second exon region of gene three in clone L4 and corresponds to nucleotides 4565 to 7216 in
25 the sequence of clone L4 was isolated. This fragment was made blunt ended with Klenow and cloned into the binary transformation vector PAL1001 previously described. PAL1001 was first cut with HindIII and made blunt ended with Klenow. Clones containing this fragment
30 (promoter/first exon/intron/partial second exon) were recovered. A clone was chosen that contained this fragment in the proper orientation such that the direction of transcription was towards the nos ter in PAL1001. This vector was named PAL1421. This vector
35 contains approximately 1.9kb of upstream promoter region from the gene 3 in clone L4 followed by the first exon, the complete intron and 15 bases of the second exon of gene three followed by a polylinker containing the

following unique sites: SalI, XbaI, BamHI, SmaI, KpnI, SstI, and finally the nos ter polyadenylation signal. A variant of this vector was constructed by digesting PAL1421 with EcoRI and isolating the fragment from this clone that contains the promoter polylinker nos ter sequences but contained less of the upstream region of the promoter. This fragment was re-cloned into PAL1009. PAL1009 is a Bin19 derived vector from which most of the polylinker has been removed. This vector was constructed by digesting Bin19 with HindIII and SstI, making these sites blunt ended with Klenow and relegating such that a vector was recovered that contained a single unique EcoRI site for the insertion of fragments. PAL1009 was digested with EcoRI and the EcoRI fragment from PAL1421 that contains a shorter promoter/exon/intron/second exon/polylinker/nos ter structure was added to it. This gave rise to the vector PAL1422, a vector that is essentially the same as PAL1421 with the exception that there is less 5' promoter region. It should be noted that both PAL1421 and PAL1422 contain the intron from the third gene. For constructs which the presence of the intron may not be desired, intron sequences were removed from PAL1421 by first digesting PAL1421 with EcoRI and replacing the promoter/exon/intron/second exon/polylinker/nos ter structure with the promoter/polylinker/nos ter structure from pPAL0420 using EcoRI such that a longer 5' promoter region is reconstructed in the binary transformation vector. The resultant vector was named PAL1423. The outline of this construction is shown in Figure 7d.

In Figure 7e, a schematic diagram of the relationship of the above described vectors is presented. It should be noted that the vectors outlined in this Figure fall into three categories: 1) vectors which contain 5' upstream promoter regions that are substantially derived from the upstream region of the gene Bp4C (pPAL0420, PAL1420, PAL1423), 2) promoter constructs that contain 5' upstream promoter regions and intron sequences from the

gene Bp4C (PAL1422, PAL1421) and, 3) promoters which contain a chimeric 5' upstream region in which a portion of the 5' DNA sequence is inverted relative to the arrangement which appears in the genomic clone and uses the promoter fragment of Bp4A as a core promoter structure (PAL1107, PAL1106). It should be noted that the functioning of each of these constructs can vary from plant species to plant species and it may be desirable to test a number of these promoter constructs when carrying out certain aspects of this invention.

The construction of pollen specific vectors that utilize the promoter regions of clones L10 and L19 was conducted as follows. The construction of the pollen specific vectors depicted in Figure 8 utilizes promoter regions from clone L10. The start of transcription of clone L10 is located at nucleotide 1. The ATG start codon is located at nucleotides 45-47. The promoter region of this clone was excised by first subcloning the EcoRI - XbaI fragment of the clone that encompasses the entire promoter region and a portion of the first exon (the XbaI site is nucleotide 358 in the DNA sequence). This subclone (pPAL10EX) was then digested with HincII and NdeI. The NdeI site is located immediately downstream of the ATG start codon at nucleotide 60 and the HincII site is located at nucleotide number -399. The digestion with these two enzymes releases a DNA fragment of 459 nucleotides which contains 62 nucleotides of untranslated transcribed leader sequence, and 397 nucleotides of 5' promoter region. The NdeI site in this fragment was made blunt ended by the use of Klenow, and this fragment was subcloned into the HincII site of the polylinker of pGEM 4Z. Clones were recovered in both orientations and the clone that contained the fragment in the orientation: HindIII, SphI, PstI, HincII, promoter-62 base pair leader fragment (NdeI blunt/HincII, does not cut with either HincII or NdeI) XbaI, BamHI, SmaI, KpnI, SstI, EcoRI was chosen and named pPAL1020. To add additional upstream regions, the HincII - HincII fragment

that is approximately 1 Kb in length and is immediately upstream of the HincII site at position 391 in the DNA sequence was isolated from pPAL10EX by digestion with HincII and gel elution of this fragment. This HincII fragment was cloned into the SmaI site of pGEM 4Z. Clones which contained the fragment in both orientations were recovered and a clone that contained the fragment in the following orientation was chosen: HindIII, SphI, PstI, HincII, SalI, XbaI, BamHI, the HincII fragment in the same orientation as in the genomic clone, that being right to left, 5'-3' (as a HincII/SmaI insertion which does not cut with either enzyme), KpnI, SstI, EcoRI. This subclone (pPAL10Hc) was digested with Kpn I, made blunt end by the use of Klenow, then digested with EcoRI. To this cut subclone was added the promoter/untranslated leader sequence of pPAL1020 by digesting pPAL1020 with HincII and EcoRI, and adding this promoter fragment to the cut pPAL10Hc. The resultant subclone contained a reconstructed promoter region of clone L10 differing from the intact region by only the filled in KpnI site used for the joining of the two promoter fragments. This construct was named pPAL1021. This vector contains in the following order: HindIII, PstI, SphI, HincII, SalI, XbaI, BamHI, the approximately 1 Kb HincII fragment joined to the HincII - NdeI promoter fragment followed by XbaI, BamHI, SmaI, KpnI, SstI, and EcoRI. This subclone allows for the convenient removal of the promoter region of clone L10 such that the promoter can be easily used in cassette transformation vectors. The outline of this construction is shown in Figure 8. The promoter region of pPAL1021 was used for the construction of a pollen specific cassette transformation vector by carrying out the following constructs: The plasmid pPAL1021 was digested with NcoI and PstI. The plasmid was treated with Klenow and relegated. This procedure effectively removed the portion of the polylinker that was 5' to the promoter in pPAL1021. This plasmid was then digested with HindIII and SstI, and cloned into the HindIII and

SstI sites of PAL1001, giving rise to PAL1121. PAL1121 has in the following order: the promoter essential for pollen development and/or function of clone L10 (approximately 1.1-1.2 Kb), followed by a polylinker with the following unique sites: XbaI, BamHI, SmaI, KpnI, SstI, followed by the nos ter. The construction of this is outlined in Figure 8. The L10 promoter isolated here is also referred to as promoter Bp10. Also see Albani, D., Sardana, R., Robert, L.S., Altosaar, I., Arnison, P.G., and Fabijanski, S.F., A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *Plant J.* 2: 331-342 (1992).

The promoter region of the clone L19 was also used for construction of pollen specific vectors. The construction of these vectors is as shown in Figure 9. Clone L19 has a single pollen specific gene contained with it. The start of transcription in this gene is located at position 1 in the DNA sequence. The ATG start codon is located at nucleotide position 136-138. The only intron is located at nucleotides 1202-1387, the stop translation codon is located at nucleotides 2024-2026. The end of transcription is located at approximately nucleotide 2074. The entire EcoRI fragment of this clone was subcloned into PGEM 4Z by using the EcoRI site located in the polylinker. The resultant clone was named pPAL1901. The promoter region of this clone was excised as a single fragment by digesting pPAL1901 with BamHI and EcoRV, and a 2177 basepair fragment corresponding to the promoter region was isolated. This fragment covers from nucleotide -2200 (BamHI) to nucleotide 156 (EcoRV). This promoter fragment contains over 2Kb of 5' upstream region of the promoter in clone L19, 134 basepairs of 5' T h e promoter region of the clone L19 was also used for construction of pollen specific vectors. The construction of these vectors is as shown in Figure 9. Clone L19 has a single pollen specific gene contained

with it. The start of transcription in this gene is located at position 1 in the DNA sequence. The ATG start codon is located at nucleotide position 136-138. The only intron is located at nucleotides 1202-1387, the stop translation codon is located at nucleotides 2024-2026. The end of transcription is located at approximately nucleotide 2074. The entire EcoRI fragment of this clone was subcloned into PGEM 4Z by using the EcoRI site located in the polylinker. The resultant clone was named pPAL1901. The promoter region of this clone was excised as a single fragment by digesting pPAL1901 with BamHI and EcoRV, and a 2177 basepair fragment corresponding to the promoter region was isolated. This fragment covers from nucleotide -2200 (BamHI) to nucleotide 156 (EcoRV). This promoter fragment contains over 2Kb of 5' upstream region of the promoter in clone L19, 134 basepairs of 5' untranslated leader sequence and 20 basepairs of translated sequence. The BamHI site in this fragment was made blunt ended by the use of Klenow and cloned into PAL1001. This step was accomplished by cutting PAL1001 with HindIII, making this site blunt ended by the use of Klenow and inserting the blunt ended BamHI - EcoRV fragment in such an orientation that the promoter was oriented 5' to 3' with respect to the polylinker/nos ter polyadenylation signal. This vector was named PAL1920 and contained within it in the following order: The promoter from clone L19 containing 134 base pairs of 5' untranslated leader sequence, 20 base pairs of translated sequence fused to a polylinker containing a former HindIII site inactivated by blunt ending, SphI, PstI, SalI, HincII, XbaI, BamHI, SmaI, KpnI, SstI (the unique cloning sites are underlined), the nos ter polyadenylation signal. This vector is convenient for the insertion of DNA sequences to be transcribed in pollen cells. The outline of this construct is shown in Figure 9. A plasmid for future construct downstream of promoter TA39 was constructed as follows: TA39 promoter

was cut out and isolated from pPHP1481 as a PstI fragment.

It was ligated into pPHP585 (i.e., BlueScript SK+) cut with PstI and NotI, together with the NotI-PstI fragment from pPHP1428, and screened for the construct containing both the PstI and PstI-NotI fragments. The screening employed in addition to PstI-NotI, PvuII, and HindIII digests. Resulting plasmid was pPHP1428. That portion of pPHP1494 incorporated into the IamS construct pPHP5840 (fig. 25) and the IamH construct pPHP5841 (fig. 26) was the HindIII -BamHI fragment containing the TA39(883) promoter and 6'.

In Figure 10, the restriction map of a *Brassica napus* genomic clone (HP101) that contains a constitutively expressed gene is shown and the fragment of this clone that contains a 5' promoter region along with a portion of transcribed sequence is identified. This fragment was isolated by first cloning the small 2.5 kb EcoRI fragment in pGEM 4Z, and obtaining a subclone that had this fragment inserted in the indicated orientation relative to the polylinker of pGEM 4Z. This clone was called pPAL0101 and was deposited January 26, 1990 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD, 20852, USA as pPAL0101/E. coli strain DH5 alpha under accession number ATCC 68210. This E. coli strain grows on standard E. coli media (LB) with 100 micrograms per ml of ampicillin. This subclone, pPAL0101, was then digested with EcoRI, treated with Klenow fragment, then digested with BamHI, which releases the promoter/transcribed region indicated. This fragment was cloned into HincII - BamHI cut pGEM 4Z, resulting in the subclone pPALHP101. The subclone can be used for the isolation of promoter sequences in vector constructs that utilize a constitutive promoter to synthesize pollen specific anti-sense RNA.

In Figure 11 a schematic representation of producing a polylysine coding gene is shown. In this construct, the cloning vector pGEM 4Z was used as a recipient of a

synthetic oligonucleotide containing a ATG start codon and to this was added a polynucleotide consisting of solely one nucleotide. This gene therefore, depending on the nucleotide used is a gene that has predominantly one codon, and codes for a protein that is composed of a polyamino acid. The construct was carried out in the following fashion: To provide a ATG start codon in a favorable initiation context, a synthetic oligonucleotide was constructed and inserted into pGEM 4Z between the HindIII and SphI sites. This nucleotide had the sequence: 5'-ACGTGGATCCAAGATGACATG-3'. The resultant subclone therefore had the DNA sequence (restriction site for a introduced BamHI site is underlined, the ATG start codon in bold) AACGT GGATCC AAG **ATG** ACA TGC GCA ACA TGG at the 5' region such that there was a ATG start codon in a favorable initiation context and a BamHI site upstream of this site for excision of the coding sequence. This subclone was digested with PstI, divided into two aliquots, one was tailed with T residues using terminal transferase and TTP, one was tailed with A residues using terminal transferase and dATP. The two tailed plasmids were mixed together, extracted with phenol-chloroform, ethanol precipitated and resuspended. The plasmid mixture was cut with SstI, and relegated. Clones that were recovered were either clones that contained on the coding strand poly A or poly T. Clones were cut with BamHI, the size of the insert determined by gel electrophoresis, and sequenced to determine if the clone coded for poly-lysine (poly A) or poly-phenylalanine (poly T, poly U in the mRNA). A clone that coded for poly-lysine and was approximately 300 nucleotides was chosen and called pPAL pLys. This clone was cut with XbaI, blunt ended with Klenow and a universal translation terminator (available from Pharmacia PL Biochemicals, Montreal, Canada) was added to it, completing the construction of the gene. This construction is shown in Figure 10.

In Figure 12 a scheme for the production of an anti-sense gene specific to the intron region of clone L19 and the production of an intronless version of the number L19 gene is shown. For the first step in this, a restriction fragment from clone L19 was isolated that is substantially all intron. This fragment was isolated by using the restriction enzyme DdeI which cuts at a number of sites in the genomic clone, but the sites at nucleotides 1186 and 1348 give rise to a restriction fragment that is substantially intron sequences, having only approximately 16 nucleotides at the 5' side of the intron that are included in the final transcript, and 10 nucleotides at the 3' side of the intron that are included in the final transcript. This DdeI fragment was isolated by gel electrophoresis, made blunt ended and cloned into SmaI cut pGEM 4Z. Clones in both orientations were obtained, and the clone that contained the intron region in the orientation: HindIII, SphI, PstI, SalI, XbaI, BamHI (former SmaI) 3' end of the intron, intron sequences, 5' end of the intron, (former SmaI), KpnI, SstI, EcoRI was chosen. This clone was named pPAL1914 and was digested with BamHI and SstI, and inserted into PAL1920 previously cut with BamHI and SstI, creating the vector PAL1954.

To create a specific restorer vector, the cDNA clone 19 cDNA (Figure 6) was fused to the promoter region as follows: The cDNA clone was digested with EcoRV and SmaI as shown in Figure 11. To this cut vector was added the EcoRV - SmaI fragment from clone L19. Clones were recovered that contained the reconstructed 5' region of the promoter and coding sequence, but carried coding sequences that lacked the intron, most of the coding regions being derived from the cDNA clone. This clone was digested with EcoRI, made blunt ended with Klenow, then digested with SmaI. A DNA fragment that represents the entire coding region and a portion of the promoter region was isolated and cloned into PAL1920 cut with SmaI, leading to a reconstruction of the promoter region

and coding region lacking an intron. This vector was called PAL1955.

In Figure 13, the production of clones containing the coding sequences of a protein functionally related to the ricin A chain protein isolated from Ricinus communis is detailed. This was accomplished by first isolating a genomic clone homologous to ricin from a genomic library of Ricinus zanzabarens DNA constructed in the vector lambda gt10 using standard protocols. The library was screened with a DNA probe that corresponds to the N-terminal leader sequence of the ricin gene. The probe sequence was obtained from the published sequence of a Ricinus communis ricin gene (Halling, et al., Nucl. Acids Res 13:8019-33, 1985). A genomic clone was isolated that contained the leader sequence and a portion of the A chain was isolated and called RIC 1B. This clone contained the promoter region, the 5' untranslated region, the N-terminal leader sequence and coding region that extended to amino acid 191(11e) in the published sequence (Halling, et al., Nucl. Acids Res 13:8019-33, 1985). The difference between the published sequence and RIC 1B was that the published nucleotide sequence at the region of 11e 191 was: (11e 191 underlined) ACG AGA ATT CGG which codes for the amino acids: The Arg 11e Arg while in RIC 1B the nucleotide sequence is ACG AGA ATT CGG, which codes for the same amino acids (The Arg 11e Arg), the only difference being the last Arg is coded for by CGG in RIC 1B while in the published sequence it is coded for by AGG. This single nucleotide substitution has the effect of introducing a EcoRI site at 11e 191. The clone RIC 1B therefore was missing the amino acids present after 11e 191 since the clone was isolated as a single EcoRI fragment. This truncated version of ricin was used for construction of ricin A chain N-terminal deletions as follows: The clone RIC 1B was digested with HincII and EcoRI. The fragment was cloned into EcoRI-SmaI cut pGEM 4Z. The resulting clone, pPALR1B was digested with PstI and BamHI. This cut clone was

digested with Exo III nuclease, treated with S1 nuclease and Klenow fragment and then relegated. Subclones were obtained that had various portions of the 5' region deleted, and some of these deletions were sequenced. One deletion, named pPAL-Ridefin had the majority of the N-terminal leader DNA sequence removed and had HindIII and SphI sites 5' to this region such that the DNA sequence was as follows: AAGCTT GCATGC GCA ACA TGG.... wherein the first six nucleotides code for a HindIII site found in pGEM 4Z, the next six nucleotides codes for the SphI site in pGEM 4Z and the following three triplets code for amino acids -20, -19, -18 (Ala The Trp...) in the published sequence (Halling, et al., Nucl. Acids Res 13:8019-33, 1985). This subclone therefore had a deletion that removed the first 15 amino acids of the A chain leader sequences of ricin. To provide a ATG start codon in a favorable initiation context, a synthetic oligonucleotide was constructed and inserted into the subclone between the HindIII and SphI sites. This nucleotide had the sequence: 5'-ACGTGGATCCAAGATGACATG-3'. The reconstructed clone (pPAL Rictr) therefore had the DNA sequence (restriction site for a introduced BamHI site is underlined, the ATG start codon in bold) AACGT GGATCC AAG **ATG** ACA TGC GCA ACA TGG at the 5' region such that there was a ATG start codon in a favorable initiation context and a BamHI site for excision of the coding sequence. The clone pPAL Rictr was digested with EcoRI, end filled with Klenow, and dephosphorylated with alkaline phosphatase. To the vector was added the universal translational terminator purchased from Pharmacia-PL biochemicals (Montreal, Canada) to provide a termination codon. The coding region from this clone was isolated by digestion with BamHI and pVUII, releasing the coding region and a small portion of pGEM 4Z, and this fragment can be cloned into the BamHI and SmaI sites of transformation vectors. This DNA fragment codes for a version of a ricin A chain in which a C-terminal portion has been deleted. It should be noted that a

number of C-terminal and N-terminal deletions of the ricin A chain have been tested *in vitro* for toxicity, and these reports have concluded that the N-terminal half of the ricin A chain is sufficient for cytotoxicity *in vitro* (for example see: Sudan et al., Nucl. Acids Res, 17:1717-32, 1989). In order to obtain a complete coding region for the ricin A chain, synthetic version of the rest of the A chain was synthesized using the published sequence. This synthetic portion of the gene extended the DNA sequence to nucleotide 1182 in the published sequence and had a EcoRI sites at both ends that allowed for the joining of this fragment to the EcoRI site at the amino acids numbers 190-192. This reconstructed version of the gene also had a BamHI site after the stop codon such that the DNA sequence of the gene at the 3' end was as follows: (nucl. 1181 is marked *) CCT CCA* TAA GGATCC GAATTC coding for amino acids: Pro Pro stop. followed by BamHI and EcoRI, the EcoRI site being used for the insertion of the synthetic portion of the gene, the BamHI being used for excision of the complete ricin A chain sequence since a synthetic BamHI site is at the 5' end of the coding region also. The clone was called pPAL Riccom.

In Figure 14 the results of anti-sense RNA inhibition of Beta-glucuronidase gene activity is shown in a histogram of GUS gene activity found in transgenic plants that contained a sense GUS gene and were retransformed with a vector containing an anti-sense GUS gene. GUS activity levels were expressed as a percentage of the GUS activity found in the original GUS+ plant TTR-48. Tissue was obtained from young (Y), medium (M) and old (O) leaves and GUS activity was assayed spectrophotometrically. Lanes 1-5, samples from the retransformed plants TTR-1 to TTR-5 (TTR-48/PAL1302); lane 6, samples from plant TTR-48; lane 7, samples from TTR-88 (TTR-48/pVU1011) and lane 8, samples from untransformed tobacco.

Figure 20 illustrates hybrid seed production using a variation of the binary cryptotoxicity method and a herbicide resistance gene. Genes 1 and 2 can be the IamS and IamH genes. The herbicide resistance gene can be any resistance gene against any herbicide. Parent A is produced with the three genes G1, G2, and H being linked by virtue of introduction on one recombinant DNA molecule, via transformation of a plant cell. It is maintained by crossing with a non-transformed isogenic strain and spraying for herbicide resistance. Parent B is homozygous for a male sterility restoration gene such as anti-sense for gene 1 and/or gene 2 and for a linked herbicide resistance gene. The resulting hybrid seed is essentially 100% herbicide resistant and restored in respect to male fertility.

Figure 21 illustrates hybrid seed production with parent A being produced and maintained as shown in Figure 20. Parent B is homozygous to herbicide resistance. The hybrid is essentially 100% herbicide resistant.

Figure 22a illustrates the use of the binary cryptotoxicity method for production of a male sterile, herbicide resistant parent A. Gene 1 and gene 2 come from different transformed lines, each homozygous for the recombinant DNA molecule, with either G1, or G2, or both, being linked to a herbicide resistance gene. The resulting parent A is essentially 100% male sterile with G1 and G2 on different chromosomes. Parent A is herbicide resistant.

Figure 22b illustrates the method used to maintain parent A shown in Figure 22a.

Figure 22c illustrates the crossing of parent A shown in Figure 22a with parent B. Parent B is the result of a transformation where the restorer gene (antisense sequences, or *lexA*, for example) and the herbicide resistance gene are linked on one recombinant DNA molecule. The result of crossing parent A and parent B is essentially a hybrid that is restored in regard to

male fertility and is herbicide resistant. If restoration to male fertility is not desired, parent B is transformed with a recombinant DNA molecule containing the herbicide resistance gene, but not the gene(s) responsible for restoring fertility.

Example 1

In this example, anti-sense RNA was used specifically for the inhibition of gene activity in plants. A tobacco plant expressing the Beta-glucuronidase gene under the control of the CaMV 35S promoter was produced by transformation of a non-transformed control tobacco cultivar, N. tabaccum, cv. Delgold. To accomplish this, tobacco leaves less than 8 inches in length were surface sterilized by exposure to ethanol for 5-6 seconds, then subsequent exposure to 1% sodium hypochlorite for a few minutes, usually 5-10 minutes, or until the cut edge of the petiole turned white, then rinsed several times in sterile distilled water. Leaf segments of approximately 0.5 to 1.0 square centimeters were cocultured for two days with Agrobacterium tumefaciens GV 3101 carrying the Ti plasmid pMP90 to provide vir functions in trans (described by Koncz, C. and Schell J., 1986, Mol. Gen. Genet. 204:383-396) carrying the binary vector pBI121.1 on shoot inducing media. This vector is a derivative of Bin19 which contains the GUS gene driven by the CaMV 35S promoter and terminated by the nos ter and is available from Clontech Laboratories, Palo Alto, CA., USA. Transformed tobacco cells were selected on a shoot-inducing medium containing 0.8% agar, MS salts, B5 vitamins, 3% sucrose, 1 mg per L of benzyladenine, 0.1 mg per L of alpha naphthalene acetic acid, (NAA) 300 ug/ml kanamycin and 500 ug/ml carbenicillin (essentially as described by Horsch et al., 1985, Science, 227:1229-31). Regenerated shoots were then transferred to a root-inducing medium consisting of B5 medium with 2% sucrose, 500 ug/ml carbenicillin and 0.5 mg/L each of NAA and indoleacetic acid (IAA). Following selection on

kanamycin, a tobacco transformant which displayed relatively high constitutive levels of GUS activity and contained a single unrearranged insertion of the 35S CaMV promoter-GUS-noster construct was selected. This plant
5 (TTR-48, GUS+) was then re-transformed with a binary vector PAL1302 which contains an anti-sense GUS gene, the construction of which is described in Figure 1. In experiments involving the retransformation of the TTR-48 tobacco plant with PAL1302, the shoot-inducing medium
10 contained 20 ug/ml hygromycin and 300 ug/ml kanamycin to insure the selection of plants containing both the sense and anti-sense GUS constructs. Transformants were grown to maturity and self-pollinated in the greenhouse.

The leaves of tobacco plants resulting from the retransformation of TTR-48 (GUS+) with the anti-sense GUS
15 vector PAL1302 were assayed for GUS activity. The GUS activity in leaf extracts was assayed spectrophotometrically. Approximately 0.5 g of leaf tissue were ground with a Polytron in 2 ml of GUS
20 extraction buffer (50 mM NaPO₄ pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 10 mM B-mercaptoethanol, 1 mM p-nitrophenyl glucuronide, 100 ug/ml bovine serum albumin, 0.02% sodium azide) incubated at 30°C for 6 h and the reaction stopped with the addition of 0.4 ml of 2.5M 2-amino-2-methyl-1,
25 3-propanediol. The amount of p-nitrophenol produced was calculated by measuring the absorbance at 415 nm. A stopped reaction containing an identical amount of leaf extract was used as a blank. Relative enzyme activities in the extracts were calculated and expressed as
30 nanomoles of p-nitrophenol produced per mg of protein per minute. Ten plants were screened and they all displayed a large reduction in GUS activity levels relative to those observed in TTR-48. Five plants (TTR-1 to TTR-5) were chosen for a detailed analysis of the effects of
35 anti-sense RNA inhibition of GUS gene activity. The retransformed tobacco plants TTR-1 to TTR-5 (Figure 14, lanes 1-5) all showed a considerable to complete (lane 4) reduction in GUS activity regardless of the developmental

stage of the leaves examined. Comparison of the highest level of GUS activity observed in the original GUS+ plant TTR-48 (Figure 14, lane 6) with the highest level found in any of the plants retransformed with PAL1302 (lanes 1-5) shows that the reduction in GUS activity was at least 90%. The levels of GUS activity found in the control plant TTR-88 (Figure 14, lane 7) were similar to those of the original GUS+ plant (lane 6) indicating that the retransformation and regeneration process undergone by TTR-48 were not responsible for the decline in GUS activity observed in TTR-1 to TTR-5. Western blot analysis of total protein extracts obtained from young leaves were performed. For extraction of leaf proteins, approximately 100 mg of tissue were ground in a 1.5 ml Eppendorf tube containing 0.7 ml of GUS extraction buffer described above. An equal volume of SDS PAGE 2X sample loading buffer (1.3 M Tris-Cl pH 6.8, 2% B-mercaptoethanol, 50% glycerol, 5 mM EDTA, 0.1% bromophenol blue) was added and the samples incubated at room temperature for 15 min. The extract was centrifuged at 12,000 rpm for 2 min and the supernatant was frozen at -80°C until use. Proteins were resolved in 10% SDS PAGE gels and immediately transferred electrophoretically onto a nitrocellulose filter. The GUS protein was then detected in the gels using the rabbit anti-B-glucuronidase antibody obtained from Clontech Laboratories and an anti-rabbit IgG alkaline phosphatase conjugate kit (Promega Biotech, Madison WI, USA) according to the manufacturers instructions. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), transferred to nitrocellulose and cross-related with antibodies specific to the GUS protein. The amount of GUS protein detected on the Western blot correlated well with the GUS activity found in the leaves of all the tobacco plants examined whether these displayed high levels of activity such as TTR-48 or no discernible activity as in TTR-3 (Figure 14, lane 3). The reduction

in GUS activity in TTR-1 to TTR-5 can therefore be directly attributed to lower quantities of GUS enzyme within these plants. Southern blot analyses were performed to confirm that the sense GUS gene was still present and intact in TTR-1 to TTR-5 and to verify that the anti-sense GUS construct had correctly integrated in the DNA of these plants. It was found that the arrangement of the original sense GUS gene in TTR-48 was unaffected by the transformation with the anti-sense gene containing vector, and it was also further ascertained that amongst the plants selected for this analysis; between 1-3 copies of the anti-sense gene were inserted into the genome of the plant which contained the sense gene. It was also determined that a single inserted anti-sense gene could lead to a near total reduction in sense gene activity. Northern blot analyses were conducted on total leaf RNA to determine if the reduction in the amounts of GUS enzyme observed in TTR-1 to TTR-5 correlated with their levels of GUS mRNA. Total RNA was prepared from tobacco leaves by grinding 0.5 g of tissue in 2 ml of extraction buffer (6 M guanidine-HCl, 0.1 M NaAc pH 6.0, 1.0% b-mercaptoethanol) for 10-30 sec using a Polytron. The mixture was then centrifuged at 5,000 rpm for 3 min, the supernatant layered on an equal volume of 5.7 M CsCl in 0.1 M NaCl, 10 mM Tris-Cl pH 7.5, 1mM EDTA and centrifuged again at 35,000 rpm for 16 h at 15°C. The resulting RNA pellet was resuspended in 0.1 M NaAc pH 6.0, 0.1% SDS and extracted with an equal volume of phenol-chloroform (50:50, v/v). The aqueous phase was adjusted to 0.3 M NaAc and the RNA precipitated with 2 volumes of ethanol. Following centrifugation, the pellet was washed in 70% ethanol and resuspended in sterile distilled water. RNA samples were resolved in the presence of methylmercuric hydroxide in 1.3% agarose gels and transferred onto a nylon membrane. The membranes were probed with (³²P) UTP labelled sense or anti-sense GUS RNA transcripts. These transcripts were made from pGEM-GUS, a plasmid obtained by inserting the BamHI-SstI

fragment of pBI221.1 (which contains the entire GUS coding sequence) into the BamHI-SstI sites of pGEM-4Z. Probes were made by cutting pGEM-GUS with EcoRI and then using T7 RNA polymerase to provide a transcript which can hybridize to anti-sense GUS RNA or by digestion with HindIII and transcription with SP6 RNA polymerase giving an anti-sense transcript that can hybridize to sense GUS mRNA only. A radiolabelled sense-specific GUS RNA probe demonstrated that the levels of GUS mRNA found in TTR-1 to TTR-5 were considerably lower than those observed in TTR-48, the original GUS+ plant. As predicted, untransformed tobacco did not possess the GUS transcript. The levels of sense mRNA correlate well with the amount of GUS protein and activity observed in these plants. Northern blot analysis using an anti-sense-specific GUS RNA probe demonstrated the presence of anti-sense GUS transcript in the retransformed plants. The reduced amounts of GUS protein and GUS activity observed in TTR-1 to TTR-5 can thus be attributed to the low levels of GUS mRNA found in these plants. Low levels of GUS mRNA were always associated with the presence of the anti-sense GUS RNA. These results clearly indicate that a target sense gene can be successfully inhibited using anti-sense RNA.

Example 1A

This example describes a method for isolating microspores in order to obtain genes that are essential to pollen formation and/or function. Microspores may be conveniently isolated by manual dissection of buds to remove anthers that are subsequently disrupted by gentle grinding in a mortar and pestle in 10 % sucrose. The extract is then filtered through a 44 um nylon mesh and the microspores collected by centrifugation at 3000 x g for one minute. The pelleted microspores are re-suspended in 10 % sucrose, filtered and pelleted as before. Other methods of isolating microspores can also be used.

Tissues other than microspores can be disrupted by a variety of methods and the disrupted tissue used for RNA isolation. It is convenient to disrupt the tissue by using a motor driven homogenizer with 10 mls of a solution of 6M guanidinium HCl, 0.1 M Na acetate, pH 6.0, 0.1 M beta-mercaptoethanol per gram of tissue. The homogenate is cleared by centrifugation at 5,000 x g and the supernatant is layered over a solution of 6M CsCl in Tris-EDTA buffer (TE buffer). Centrifugation at 100,000 x g for 12-20 hrs at 15°C is used to pellet RNA which is subsequently re-suspended in water and re-precipitated in the presence of 0.3 M Na acetate and two volumes of ethanol. RNA is recovered by centrifugation and re-suspended in water. The RNA obtained by such method can be fractionated by oligo-d-T cellulose chromatography to separate the polyadenylated mRNA from the bulk of the non-polyadenylated RNA. The microspore RNA is isolated by using a tight fitting motor driven glass homogenizer to disrupt the microspores. The homogenization of 300 ul of packed microspores is conducted in 1 ml 6M guanidinium-HCl, 0.1 M Na acetate, pH 6.0, 0.1 M beta-mercaptoethanol. The homogenate is centrifuged at 5000 x g and the cleared supernatant is layered over a solution of 6M CsCl in TE buffer. An overnight centrifugation at 100,00 x g is used to pellet the RNA

which is subsequently re-suspended in water and re-precipitated in the presence of 0.3 M Na acetate and two volumes of ethanol. Other methods of RNA extraction can be used to obtain the RNA from the tissues described. Standard methodology using oligo-dT cellulose is used to obtain polyadenylated mRNA from these total RNA preparations.

Example 2

This example relates to the use of constitutive promoters to regulate the expression of pollen specific anti-sense RNA. The CaMV 35S promoter from pBl 221 was isolated as a HindIII - XbaI fragment and cloned in PAL1001 previously cut with HindIII and XbaI. This produced a vector that had the CaMV 35S promoter joined to the nos ter and in between the promoter and terminator were unique sites for : XbaI BamHI, SmaI, KpnI and SstI. This vector was named PAL1007. PAL1007 was digested with BamHI and to this digested vector was added a 2.4 Kb BamHI fragment containing a coding region from clone L16 in the anti-sense orientation. This vector was called PAL1305 and was used to transform *Brassica napus*. Transformation was carried out either using the method described in Moloney, M.M., et al., (Plant Cell Reports (1989) 8:238-42) or, transformation can be carried out with surface sterilized stem epidermal layers. For this procedure, seeds of *B. napus* L. ssp. *oleifera* cv. Westar were sown in 'Promix' mixed with 2g/L of the slow-release fertilizer 'Nutricoate' in 8" pots. Plants were grown in the greenhouse under a 16 photoperiod (using natural and artificial lighting). For coculture and regeneration experiments stem-sections from the top three stem internodes of approximately 1.5 month old plants were used (i.e., those with elongated floral spikes and several open flowers). Intact stem-sections were surface sterilized for 30 seconds in 70% ethanol and 10 minutes in 1% sodium hypochlorite followed by three rinses in sterile distilled water. For transformation

Agrobacterium tumefaciens GV 3101 carrying the Ti plasmid pMP90 to provide vir functions in trans and the binary vector PAL1110 was grown on YEP media (which consists of 10 gm per L of Yeast Extract, 10 gm per L of Bacto-pepetone and 5 gm per L of NaCl, pH 7.0 containing 100 ugs per mL kanamycin for selection of bacterial cells that contained the binary vectors). Cells were grown from one to two days at 28C. The cells were collected by centrifugation and were resuspended at an approximate density of 10^6 - 10^7 cells per mL in liquid EL which consists of MS micro- and macro- nutrients and B5 vitamins containing 40 mg/L of FeNa-EDTA (obtained from BDH chemicals) and 3% sucrose, 10 mg/L BenzylAdenine, and 0.5 mg/L alpha naphthalene acetic acid (NAA) and 18.8 mM KNO_3 plus 20.6 mM NH_4NO_3 . Medium was solidified with 0.8% agar (Sigma) when the EL media was used for solid media plates.

The cell suspension was poured into the bottom of a sterile petri dish and sterilized stems were dissected directly in the bacterial suspensions. The segments were sectioned longitudinally into half segments and cut into approximately 5 mm sections. The dissected segments were placed on filter paper discs on solid EL media for a 3 day coculture under continuous fluorescent light (60 microeinsteins/m²/sec²) at 25C. After a 2-3 day coculture, explants were transferred to solid EL media containing 500 ug/mL carbenicillin, and 100 ug/mL bekanamycin (Sigma). Shoots formed in 4-8 weeks, sections were transferred to fresh solid EL media with carbinicillin and bekanamycin every 3-4 weeks. Shoots that formed and did not bleach were excised and rooted on PDR media (B5-with 2% sucrose and 0.5 mg/L each of NAA and IAA). In some cases, green non-regenerating callus growing on selective medium was separated from explants and transferred to fresh medium to stimulate regeneration. Transformed plants were placed in a misting chamber, and after two to four weeks transferred to the greenhouse. Plants were grown under a 16 hour

photoperiod and allowed to flower. Clonal propagation was used to increase plant lines as well as hand crossing and selection of seedlings from crossed plants on kanamycin containing media. This media consisted of 0.8% agar, one-tenth MS salts and 100ug/ml kanamycin with no sucrose in the media. Surface sterilized seeds were used. The seeds were surface sterilized by rinsing in 70% ethanol for a few seconds, soaking in 1% sodium hypochlorite for 15 minutes, followed by rinsing three times in sterile distilled water. Seeds were placed on the surface of the agar in sterile dishes and allowed to sprout. Plants which did not carry the kanamycin gene linked to the anti-sense gene bleached and died, while those that carried the antisense gene stayed green and were subsequently transferred to soil and allowed to flower.

Example 3

In this example, another pollen specific coding region was used with the vector PAL1007. In this case, a 1.3 Kb HindIII fragment from clone L19 was isolated, made blunt ended and cloned into the SmaI site of pGEM 4Z. This subclone was called pPAL1914 and was then digested with XbaI and SstI. This fragment was added to XbaI - SstI cut PAL1007, giving rise to a vector called PAL1307. This vector contains the CaMV 35S promoter fused to a coding region from clone L19 in the anti-sense orientation. This vector was used to transform *Brassica napus* as detailed in example 34.

Example 4

This example relates to the use of inducible promoters to regulate expression of pollen specific anti-sense RNA. In this example, the 1.2 Kb HindIII - PstI fragment of the D. melanogaster 70 KD heat shock protein promoter was isolated from the subclone pPW 229 (Holmgren, R. et al., 1979, Cell 18: 1359-1370) and cloned into HindIII - PstI cut pGEM 4Z. The heat shock promoter was excised as a HindIII - SmaI fragment and cloned into HindIII - SmaI cut PAL1001. This produced a

vector (PAL1009) that contains a heat shock promoter followed by a portion of the polylinker and nos ter. The SmaI site was used to clone the 1.3 Kb HindIII fragment from clone L19 following making this fragment blunt ended. The clone containing this fragment in the anti-sense orientation relative to the heat shock promoter was called PAL1403. This vector was used to transform *Brassica napus* as in example 34. Additionally, since a single EcoRI site exists at the end of the nos ter in this construct, a selection marker gene was added to this construct, the enzyme Beta-glucuronidase driven by the CaMV 35S promoter using this unique EcoRI site for the insertion of this gene. This vector, which is the same as PAL1403 except that it now contains a convenient gene for scoring transformation was named PAL1408 and was used for transformation of *Brassica napus* as described in example 34.

Example 5

This example relates to the use of a promoter essential for pollen development and/or function to specifically express pollen specific anti-sense RNA in pollen cells. For this, the vector PAL1107 was used for the production of anti-sense RNA from the cDNA clone 4F described in Figure 4. To construct this anti-sense vector, the EcoRI fragment from the cDNA clone 4F was isolated, made blunt ended with Klenow and cloned into the SmaI site of PAL1107. A vector that contained the cDNA clone in the anti-sense orientation (as determined by restriction enzyme analysis) was chosen. This vector was named PAL11074F and used to transform *Brassica napus* as described in example 34.

Example 6

For this example, the vector PAL1107 was used for the production of anti-sense RNA from the 2.4 Kb BamHI fragment of clone L16 described in example 2. To construct this anti-sense vector, the 2.4 Kb BamHI fragment from clone L16 was isolated and cloned into the BamHI site of PAL1107. A vector that contained this

fragment in the anti-sense orientation (as determine by restriction enzyme analysis) was chosen. This vector was named PAL1107-16CRAS and used to transform *Brassica napus* as described in example 34.

5 Example 7

For this example, the vector PAL1107 was used for the production of anti-sense RNA from the 1.3 Kb HindIII fragment of clone L19 described in example 3. To construct this anti-sense vector, the 1.3 Kb HindIII
10 fragment from clone L19 was isolated and made blunt ended and cloned into the SmaI site of PAL1107. A vector that contained this fragment in the anti-sense orientation (as determine by restriction enzyme analysis) was chosen. This vector was named PAL1107-19CRAS and used to
15 transform *Brassica napus* as described in example 34.

Example 8

For this, the vector PAL1107 was used for the production of anti-sense RNA from the cDNA clone related to clone L10 described in Figure 5. To construct this
20 anti-sense vector, the EcoRI fragment from the cDNA clone was isolated, made blunt ended with Klenow and cloned into the SmaI site of PAL1107. A vector that contained the cDNA clone in the anti-sense orientation (as determined by restriction enzyme analysis) was chosen.
25 This vector was named PAL110710G and used to transform *Brassica napus* as described in example 34.

Example 9

For this, the vector PAL1107 was used for the production of anti-sense RNA from the cDNA clone related to clone L19 described in Figure 6. To construct this
30 anti-sense vector, the EcoRI fragment from the cDNA clone was isolated, made blunt ended with Klenow and cloned into the SmaI site of PAL1107. A vector that contained the cDNA clone in the anti-sense orientation (as
35 determine by restriction enzyme analysis) was chosen. This vector was named PAL110719 and used to transform *Brassica napus* as described in example 34.

Example 10

In this example, the promoter essential for pollen development and/or function in the vector PAL1421 was used for the production of anti-sense RNA using the cDNA clone homologous to the gene contained with L10. This was accomplished by excising the cDNA clone from the cloning vector with EcoRI and making this fragment blunt ended with Klenow. This blunt ended cDNA fragment was cloned into the SmaI site of PAL1421. Clones were recovered that contained the cDNA insert in both orientations, and one was chosen that contained the insert in the anti-sense orientation relative to the promoter of PAL1421, resulting in the formation of a binary transformation vector PAL1492. PAL1492 was used to transform *Brassica napus* as described in example 34.

Example 11

In this example, the promoter essential for pollen development and/or function in the vector PAL1121 was used for the production of anti-sense RNA using the cDNA clone homologous to the gene contained within L10. This was accomplished by excising the cDNA clone from the cloning vector with EcoRI and making this fragment blunt ended with Klenow. This blunt ended cDNA fragment was cloned into the SmaI site of PAL1121. Clones were recovered that contained the cDNA insert in both orientations, and one was chosen that contained the insert in the anti-sense orientation relative to the promoter of PAL1121, resulting in the formation of a binary transformation vector PAL1110. PAL1110 was used to transform *Brassica napus* as described in example 34.

Example 12

In this example, the promoter essential for pollen development and/or function from clone 19 was used for the expression of pollen specific anti-sense RNA. The transformation vector PAL1920 was digested with SmaI. To the SmaI site was added the DNA fragment corresponding to the cDNA clone homologous to clone L10 by digesting the vector containing this clone with EcoRI and making the

fragment blunt ended. This blunt ended cDNA fragment was cloned into the SmaI site of PAL1920. Clones were recovered that contained the cDNA insert in both orientations, and one was chosen that contained the insert in the anti-sense orientation relative to the promoter of pPAL1920, resulting in the formation of a binary transformation vector PAL1921. PAL1921 was used to transform *Brassica napus* as described in example 34.

Example 13

In this example, anti-sense RNA is made specifically to the intron region of clone L19 and a specific restorer gene is made which lacks the intron from clone L19. The construction of these two vectors is outlined in Figure 12. PAL1954 was used to transform *Brassica napus* as in example 34 to make a male sterile line. To create a specific restorer plant line, the vector PAL1955 was used to transform *Brassica napus* as in example 34.

Example 14

In this example, the highly active promoter fragment of clone HP101 we used to synthesize anti-sense RNA to the intron region of clone 19. For this example, the subclone containing the intron region of clone 19 that is detailed in Figure 12, pPAL1914 was digested with HindIII and BamHI. The promoter fragment from clone pPALHP101 was added to this construct as a HindIII - BamHI fragment giving rise to clones containing the promoter fragment in the anti-sense orientation relative to the intron. This clone contained the promoter in an orientation such that transcription of the promoter would cause the production of an RNA a portion of which would contain anti-sense RNA homologous to the intron region of clone L19. This clone was called pPAL19HP. The clone pPAL19HP was digested with HindIII and SstI, and cloned into the vector PAL1001 using the HindIII and SstI sites of PAL1001, creating PALHP19. PALHP19 was used to transform *Brassica napus* as in example 34. It should be noted that the vector PAL1955 (see example 13 and Figure 12) can be used for fertility restoration in plants that carry PALHP19.

Example 15

The promoter essential for pollen development and/or function contained in the subclone pPAL0420 was used for the construction of an anti-sense RNA gene under the control of a promoter essential for pollen development and/or function as follows: A DNA fragment coding for a polyubiquitin of Arabidopsis was isolated from a plasmid which contains a polyubiquitin gene that has 5 copies of the ubiquitin monomeric protein was obtained from the University of Wisconsin, Madison, WI, USA and is described in Burke et al., Molecular and General Genetics, in press. A BamHI - BglII fragment was isolated that contains 3 of the 5 copies of the polyubiquitin gene and this fragment was inserted into pPAL0420 by using the single BamHI site of the polylinker of PAL0420. This gave rise to a plasmid containing the promoter essential for pollen development and/or function from clone L4 followed by an DNA fragment containing ubiquitin coding sequences in the anti-sense orientation followed by the nos ter polyadenylation signal. This promoter/anti-sense gene construct was excised from pPAL0420 by digestion with EcoRI. The EcoRI fragment that contains the promoter anti-sense gene was inserted into the EcoRI site of the polylinker of Bin19, resulting in the formation of a binary transformation vector was named PAL1479. PAL1479 was used to transform *Brassica napus* as described in example 34.

Example 16

The vector PAL1479 was used to transform tobacco as described in example 34.

Example 17

The promoter essential for pollen development and/or function contained in the subclone pPAL0420 was used for the construction of a pollen specific anti-sense RNA gene as follows: A DNA fragment coding for pine actin was obtained from J. Kenny-Byrne, Petawawa Canada. Two clones were obtained, Pac 1-A and Pac 2, the clone Pac 1-A being described in: Canadian Journal of Forestry

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Research (1988) 18:1592-1602, and the second clone Pac 2 (the sequence being closely homologous to that in Pac 1-A and the nucleotide sequence of which having been submitted for publication). A SphI fragment was isolated from Pac 2 that contains the complete coding sequence of pine actin. This fragment also contains a small amount of 5' and 3' non-coding region. This SphI fragment was cloned into the unique SphI site of pGEM 4Z. From this subclone, a XbaI fragment was isolated that contains only coding region and this XbaI fragment was cloned into the unique EcoRI site of pGEM 4Z. A clone was chosen that had the orientation such that the 5' end of the gene was next to the BamHI site in the polylinker and the 3' end of the gene was next to the SalI site in the polylinker. This plasmid was called pPAL PAC. The actin coding region was isolated from pPAL PAC by digestion with BamHI and SalI. This BamHI - SalI fragment was cloned into pPAL0420 using the BamHI and SalI sites contained within the polylinker of pPAL0420. This gave rise to a plasmid containing the promoter essential for pollen development and/or function from clone L4 followed by an DNA fragment containing the actin coding sequence in the anti-sense orientation followed by the nos ter polyadenylation signal. This promoter/anti-sense gene construct was excised from pPAL0420 by digestion with EcoRI. The EcoRI fragment that contains the promoter anti-sense gene was inserted into the EcoRI site of the polylinker of Bin19, resulting in the formation of a binary transformation vector PAL1498. PAL1498 was used to transform *Brassica napus* as described in example 34.

Example 18

The vector PAL1498 was used to transform tobacco plants as outlined in example 34.

Example 19

In this example, tobacco that has been previously transformed to hygromycin resistance was transformed with an anti-sense gene that specifically blocks the hygromycin resistance in the pollen cells by virtue of

the fact that the anti-sense gene is under the control of a promoter essential for pollen development and/or function in the vector PAL1106. Transformed tobacco that were resistant to hygromycin were obtained using the
5 vector PAL1302 which is described in example 1. Selection of tobacco plant cells resistant to hygromycin was via coculture with PAL1302 and selection of 50 ug per ml hygromycin. Southern blot analysis demonstrated the presence of 5-6 copies of the sense hygromycin
10 phosphotransferase gene in one plant. This plant, referred to as TTR-122, was retransformed with a vector called PAL1107A. PAL1107A is the vector PAL1106 to which has been added the 0.8 Kb BamHI hygromycin phosphotransferase fragment isolated from PAL1302 and
15 inserted into PAL1106 in the anti-sense orientation relative to the promoter essential for pollen development and/or function in PAL1106. The resulting vector was called PAL1107A. Plants obtained from this transformation were resistant to both hygromycin and
20 kanamycin in the leaf tissue and were shown by southern blot analysis to contain the anti-sense gene. These plants were allowed to grow in the greenhouse and self fertilize. Clonal propagation of these plants were used as a preliminary increase of single plants, and these
25 clonally propagated plants were used for the production of male sterile plant line. For example, a plant which contained a single copy of the anti-sense gene and was derived from the transformation of TTR-122 was planted in a sand soil mixture and allowed to grow in the
30 greenhouse. This plant is referred to as TTR-203. Measurement of the hygromycin phosphotransferase activity in this plant demonstrated high activity in leaves, petals, stigma and pistal and anther walls, but very low levels in pollen. TTR-122 showed high levels of
35 hygromycin phosphotransferase activity in leaves, petals, stigma and pistal and anther walls and in pollen. This demonstrated that the anti-sense gene was effective in blocking the expression of the sense gene only in the

pollen. Northern blot analysis confirmed the presence of the anti-sense transcript specifically in the pollen of TTR-203 and also demonstrated low levels of the sense gene mRNA. When flower buds first appeared, TTR-203 was
5 watered three times weekly with a solution of hygromycin (250 ugs per mL), thoroughly saturating the sand soil mixture. This watering was continued for approximately four weeks, or the period of time in which the major flowering was occurring. Flowers produced during this
10 time on plants containing the anti-sense gene were male sterile. Anther and pollen formation was inhibited, and mature pollen failed to develop. Female fertility was unaffected by this treatment as hand pollination could be used for the pollination of the female portion of the
15 male sterile flowers. Pollen that was from a hygromycin resistant plant was used for the hybrid seed production. Watering of the plants with hygromycin was stopped, and normal watering was resumed. Flowers that formed on plants after the hygromycin watering was stopped were
20 male fertile and set selfed seed.

Example 20

The pollen specific vector PAL1107 was used for the production of male sterile plants. The plant TTR-122 described above, was retransformed with a vector called
25 PAL1107HYGAS. Transformation was conducted as described in example 34. PAL1107HYGAS is the vector PAL1107 to which has been added the 0.8 Kb BamHI hygromycin phosphotransferase fragment isolated from PAL1302 and inserted into PAL1107 in the anti-sense orientation
30 relative to the promoter essential for pollen development and/or function in PAL1107. Plants obtained from this transformation were resistant to both hygromycin and kanamycin in the leaf tissue and were shown by southern blot analysis to contain the anti-sense gene. These
35 plants were allowed to grow in the greenhouse and self fertilize. Clonal propagation of these plants were used as a preliminary increase of single plants, and these

clonally propagated plants were used for the production of male sterile plant lines as in example 19.

Example 21

5 For the production of male sterile plants, the vector PAL1419 was used to transform tobacco as outlined in example 34. The vector PAL1419 contains the promoter essential for pollen development and/or function from clone L4 controlling the expression of the NPT II gene oriented in the anti-sense orientation relative to the
10 promoter essential for pollen development and/or function. This vector also contains a constitutive version of the NPT II gene in the sense orientation driven by the nos promoter. This vector therefore can confer resistance to kanamycin in all plant cells except
15 pollen cells wherein the expression of the sense gene is inhibited by the expression of the anti-sense gene which is specifically expressed in the pollen. Tobacco plants were obtained following transformation of the vector PAL1419. These plants were rooted and allowed to set
20 flower. Plants were watered with kanamycin while flowering.

Example 22

The vector PAL1419 was used to transform *Brassica napus* as outlined in example 34.

Example 23

The vector PAL1419 was used for the transformation of petunia leaf discs.

Example 24

5 In this example, the complete ricin A chain (pPAL Riccom) described in Figure 13 was inserted into the vector PAL1420 as a BamHI fragment. Vectors were recovered that contained the ricin gene in both the sense and anti-sense orientation. A vector that contained the ricin A chain in the sense orientation was recovered and called PAL1420RIC. A vector that contained the gene in the anti-sense orientation was called PAL1420RICAS. PAL1420RIC and PAL1420RICAS were used for transformation of *Brassica napus* as described in example 34 giving rise to plants that carried either the sense or anti-sense copy of the ricin A chain gene under the control of the promoter essential for pollen development and/or function in PAL1420.

Example 25

20 In this example, the truncated version of the ricin A chain (pPAL Rictr) described in Figure 13 was inserted into the vector PAL1420 as a BamHI fragment. Vectors were recovered that contained the truncated ricin gene in both the sense and anti-sense orientation. A vector that contained the ricin A chain in the sense orientation was recovered and called PAL1420tRIC. A vector that contained the gene in the anti-sense orientation was called PAL1420tRICAS. PAL1420tRIC and PAL1420tRICAS were used for transformation of *Brassica napus* as in example 34 giving rise to plants that carried either the sense or anti-sense copy of the truncated ricin A chain gene under the control of the promoter essential for pollen development and/or function in PAL1420.

Example 26

35 PAL1420RIC and PAL1420RICAS were used for transformation of tobacco plants as in example 34, giving rise to plants that carried either the sense or anti-

sense copy of the ricin A chain gene under the control of the promoter essential for pollen development and/or function in PAL1420.

Example 27

5 The vectors PAL1420tRIC and PAL1420tRICAS were used for transformation of tobacco plants as in example 34 giving rise to plants that carried either the sense or anti-sense copy of the truncated ricin A chain gene under the control of the promoter essential for pollen
10 development and/or function in PAL1420.

Example 28

15 For this example, the vector PAL1423 was used to express the polylysine gene contained in the subclone pPAL pLys. The coding region from pPAL pLys was isolated by BamHI digestion and cloned in the sense orientation into BamHI cut PAL1423, giving rise to PAL1487. PAL1487 was used to transform tobacco as in example 34.

Example 29

20 For this example, the vector PAL1920 was used to express the polylysine gene contained in the subclone pPAL pLys. The coding region from pPAL pLys was isolated by BamHI digestion and cloned in the sense orientation into BamHI cut PAL1920, giving rise to PAL1987. PAL1987 was used to transform tobacco as in example 34.

25 Example 30

30 In this example, a promoter essential for pollen development and/or function was used to synthesize a protein molecule that is destructive to cellular function and development, namely the protease trypsin. The cDNA sequence coding for trypsin has been described by Stevenson et al., Nucl Acids Res, 1986, 14:8307-30. The cDNA clone pMPt9 was obtained and used for the production of a modified trypsin molecule in which the N-terminal amino acid residues were removed to give a protein that
35 consisted of solely the active protease form of trypsin and differed from the mature form in that there was a methionine residue at the N-terminal position of the mature protein, replacing the Isoleucine found at this

position in the active mature protein. This was accomplished by digesting the plasmid pMPt9 with FokI and PstI, and recovering a fragment that encompasses nucleotides 81 to 835, the nucleotides after 835 being the G:C tail used for cloning of the cDNA, and treating this fragment with Klenow, and cloning into blunt ended SmaI cut M13mp19RF, and isolating a single stranded phage clone that was used for site specific metagenesis to change the isoleucine codon at nucleotides 84-86 in the published sequence from ATT to ATG, introducing a initiation codon where the isoleucine codon was. The mutated gene recovered was excised with SalI and SstI, and inserted into PAL1421 and named PAL1456. The vector PAL1456 was used to transform tobacco as in example 34. It should be noted that a restorer gene can be made if a trypsin inhibitor is inserted in an analogous fashion using a clone L4 derived promoter and transformed into a male parent line. The expression of the trypsin inhibitor in the hybrid will specifically block the activity of the trypsin enzyme. A number of cDNA and genomic DNA sequences can be found for soybean and other trypsin inhibitors, for example see: Jofuku, K.D. and Goldberg, R.B., The Plant Cell 1989, 1:1079-1093.

Example 31

In this example, we use a promoter essential for pollen development and/or function to synthesize the enzyme IamH specifically in pollen cells. The enzyme has activity that can cause the production of NAA from NAM, the substance NAA functioning as a plant hormone that is substantially toxic to developing pollen grains, while the precursor NAM being relatively non-toxic. For this example, the IamH gene was inserted into the vector PAL1423. The IamH gene was isolated from pPCV311 as described in Figure 19 and cloned as a BamHI - SstI fragment in the BamHI - SstI sites of PAL1423, creating PAL1424. This vector has the IamH gene (T-DNA gene 2) under the control of a promoter essential for pollen

development and/or function from clone L4. PAL1424 was used to transform tobacco as outlined in example 34.

Example 32

5 The vector PAL1424 was used to transform *Brassica napus* as outlined in example 34.

Example 33

10 In this example, the vector PAL1107 was used for the production of tissue-specific GUS (beta-glucuronidase) enzyme. The gene for this enzyme is available from Clontech Laboratories, Palo Alto, CA., USA. The gene was inserted into PAL1107 as a BamHI - SstI fragment and was used to transform tobacco as in example 34. Plants produced had detectable GUS activity only in developing pollen cells, and not any other tissue tested. It should
15 be noted that application of non-toxic analog of glucuronic acid to which has been conjugated a toxic molecule such as glyphosate could be applied to these plants and cleavage of the toxic moiety from the glucuronic acid would occur only in pollen cells. This
20 provides an example of an enzyme that could be used for the production, in a tissue-specific fashion, of a toxic substance from a non-toxic analog.

Example 34

25 This example describes methods used to transform tobacco and *Brassica napus*.

For tobacco transformation, the tobacco cultivar, *N. tabaccum*, cv. Delgold was used. To accomplish this transformation, tobacco leaves less than 8 inches in length were surface sterilized by exposure to ethanol for
30 5-6 seconds, then subsequent exposure to 1% sodium hypochlorite for a few minutes, usually 5-10 minutes, or until the cut edge of the petiole turned white, then were rinsed several times in sterile distilled water. Leaf segments of approximately 0.5 to 1.0 square centimeters
35 were excised from the sterile leaves, and were cocultured on shoot inducing media for two days with *Agrobacterium tumefaciens* GV 3101 carrying the Ti plasmid pMP90 to provide vir functions in trans (described by Koncz, C.

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and Schell, J., 1986, Mol. Gen. Genet. 204:383-396) carrying the binary vector of interest. The vector is usually a derivative of Bin19 which contains the NPT II gene driven by the nopaline synthase promoter and terminated by the nos ter for selection of plant cells with kanamycin. Bin19 is available from Clontech Laboratories, Palo Alto, CA., U.S.A. Transformed tobacco cells are selected on a shoot-inducing medium containing 0.8% agar, MS salts, B5 vitamins, 3% sucrose, 1 mg per L of benzyladenine, 0.1 mg per L of alpha naphthalene acetic acid, (NAA) 300 µg/ml kanamycin and 500 µg/ml carbenicillin (essentially as described by Horsch et al., 1985, Science, 227:1229-31). Regenerated shoots are then transferred to a root-inducing medium consisting of B5 medium with 2% sucrose, 500 µg/ml carbenicillin and 0.5 mg/L each of NAA and indoleacetic acid (IAA). Rooted transformants are transferred to a misting chamber containing high humidity, after which the humidity is gradually lowered and plants are subsequently transferred to the greenhouse.

For transformation of Brassica napus, the binary vector containing Agrobacterium strain GV 3101 carrying pMP90 to provide vir functions in trans is used. Transformation was carried out either using the method described in Moloney, M.M., et al., (Plant Cell Reports (1989) 8:238-42) or, transformation can be carried out with surface sterilized stem epidermal layers. For this procedure, seeds of B. napus L. ssp. oleifera cv. Westar were sown in 'Promix' mixed with 2g/l of the slow-release fertilizer 'Nutricoate' in 8" pots. Plants were grown in the greenhouse under a 16 photoperiod (using natural and artificial lighting). For coculture and regeneration experiments stem-sections from the top three stem internodes of approximately 1.5 month old plants were used (i.e., those with elongated floral spikes and several open flowers). Intact stem-sections were surface sterilized for 30 seconds in 70% ethanol and 10 minutes in 1% sodium hypochlorite followed by three rinses in

sterile distilled water. For transformation *Agrobacterium tumefaciens* GV 3101 carrying the Ti plasmid pMP90 to provide vir functions in trans and the binary vector of choice was grown on YEP media (which consists of 10 gm per L of Yeast Extract, 10 gm per L of Bactopeptone and 5 gm per L of NaCl, pH 7.0 containing 100 ugs per mL kanamycin for selection of bacterial cells that contained the binary vectors). Cells were grown from one to two days at 28C. The cells were collected by centrifugation and were resuspended at an approximate density of $10^6 - 10^7$ cells per mL in liquid EL which consists of MS micro- and macro- nutrients and B5 vitamins containing 40 mg/L of FeNa-EDTA (obtained from BDH chemicals) and 3% sucrose, 10 mg/L BenzylAdenine, and 0.5 mg/L alpha naphthalene acetic acid (NAA) and 18.8 mM KNO_3 plus 20.6 mM NH_4NO_3 . Medium was solidified with 0.8% agar (Sigma) when the EL media was used for solid media plates.

The cell suspension was poured into the bottom of a sterile petri dish and sterilized stems were dissected directly in the bacterial suspensions. The segments were sectioned longitudinally into half segments and cut into approximately 5 mm sections. The dissected segments were placed on filter paper disc on solid EL media for a 3 day coculture under continuous fluorescent light (60 microeinsteins/m²/sec²) at 25C. After a 2-3 day coculture, explants were transferred to solid EL media containing 500 ug/mL carbenicillin, and 100 ug/mL bekanamycin (Sigma). Shoots formed in 4-8 weeks, sections were transferred to fresh solid EL media with carbinicillin and bekanamycin every 3-4 weeks. Shoots that formed and did not bleach were excised and rooted on PDR media (B5- with 2% sucrose and 0.5 mg/L each of NAA and IAA). In some cases, green non-regenerating callus growing on selective medium was separated from explants and transferred to fresh medium to stimulate regeneration. Transformed plants were placed in misting chamber, and after two - four weeks transferred to the

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greenhouse. Plants were grown under a 16 hour photoperiod and allowed to flower.

Clonal propagation was used to increase plant lines as well as hand crossing and selection of seedlings from
5 crossed plants on kanamycin containing media. This media consisted of 0.8% agar, one-tenth MS salts and 100 ugs per mL bekanamycin (available from Sigma Chemicals, St. Louis, MO., U.S.A.) with no sucrose in the media. Surface sterilized seeds were used. The seeds were
10 surface sterilized by rinsing in 70% ethanol for a few seconds, soaking in 1% sodium hypochlorate for 15 minutes, followed by rinsing three times in sterile distilled water. Seeds were placed on the surface of the agar in sterile dishes and allowed to sprout. Plants
15 which did not carry the kanamycin gene linked to the antisense gene bleached and died, while those that carried the antisense gene stayed green and were subsequently transferred to soil and allowed to flower.

Example 35

20 This example describes the isolation of two genes involved in tumor formation in plant tissues following infection with Agrobacterium, the IamS and the IamH genes from the Ti plasmid of the Agrobacterium tumefaciens strain C58. The isolation of the IamH gene is
25 particularly described. The source of DNA coding for these genes was the plasmid pPCV 311. The plasmid pPCV311 is described in: Koncz, C. and Schell, J., Molecular and General Genetics, (1986), 204:383-396, and contains the oncogenic region of the T-DNA plasmid
30 contained in the C58 strain of Agrobacterium. The plasmid pPCV 311, contains a region of T-DNA that when transferred to plant cells causes tumor formation. This oncogenic region of the T-DNA is entirely contained in the plasmid pPCV-311. This region of DNA contains four
35 genes, that when expressed in plant cells are sufficient for tumor formation. The approximate coding regions of these four genes and the direction of transcription of these four genes are indicated in Figure 15. The other

portions of the vector pPCV 311 are not shown in that they are not relative to the following constructions. Additionally, the oncogenic region of the Agrobacterium strain C58 is located on the T-DNA plasmid within that bacterium, commonly referred to as the wild-type nopaline plasmid. A nearly identical oncogenic region is also found in wild type octopine strains which could also be used as a source of genes. The complete nucleotide sequence of an octopine strain oncogenic region is described by Barker et al., Plant Molecular Biology 2:335-50 (1983). The partial sequence obtained from various constructs of genes derived from pPCV 311 was compared to the published nucleotide sequence.

Two genes were isolated from pPCV 311, the IamH and the IamS genes, commonly referred to as genes 2 and 1 respectively. The IamH gene was isolated by first subcloning the indicated HindIII fragment, a fragment that contains all of the coding region of gene 2 and additional 5' sequences that were subsequently removed for the construction of a promoterless version of the gene. The restriction sites mapped in this subclone are shown in Figure 15 and the subclone is referred to as pPAL G2. For the isolation of coding sequences only, pPAL G2 was first split into two smaller clones and the gene later reconstructed. The XbaI - SmaI and SmaI - SmaI fragments shown in Figure 15 were isolated by gel elution and subsequently cloned into the following vectors: The SmaI - SmaI fragment was cloned into pGEM 4Z, giving rise to pPAL899. The XbaI - SmaI fragment was subcloned into pGEM 7Z, giving rise to pPAL898. The 5' non-coding sequences of the IamH gene that are present in this subclone were removed in the following fashion: pPAL898 was digested with EcoRI, the EcoRI site is in the promoter region of the clone, and in this subclone is the only EcoRI site. This digested DNA was then treated with Exonuclease III, and following digestion treated with S1 nuclease and the Klenow fragment of DNA polymerase I. The treated DNA was then cut with PstI and treated with

Klenow fragment in order to make the PstI site blunt. The linear, digested, blunt ended plasmid was then relegated and used to transform *E. coli* DH5-alpha according to standard protocols. Subclones were chosen, 5 sequenced and one subclone was chosen that was deleted to 8 nucleotides in front of the ATG start of translation codon. The ATG start codon was determined by comparison of the nucleotide sequence obtained from the deleted subclones to the nucleotide sequence for the octopine 10 strain described by Barker, et al., *Plant Molecular Biology* 2:335-50 (1982). The nucleotide sequences of both the 5' non-coding and the coding region were nearly identical. This subclone was named pPAL897, the ATG codon is shown in Figure 15, the direction of 15 transcription in this case would be from right to left in Figure 15. The plasmid contained the 5' half of the coding region from the *IamH* gene, with the promoter sequences deleted.

The construction of the 3' half of the *IamH* gene, 20 contained in the plasmid pPAL898 was carried out as follows. A 3' region of the gene that contains the polyadenylation signal naturally found in the gene was isolated by digestion pPAL898 with the enzymes BamHI and ApaI. The digested DNA was treated with Klenow fragment 25 to make it blunt ended and was relegated. This gave rise to the subclone pPAL896, which is a plasmid that contains the 3' half of the *IamH* gene. To reconstruct the intact *IamH* gene, pPAL896 was digested with HindIII and SmaI, and the 3' half gene fragment was isolated by gel 30 elution. pPAL897 was digested with SmaI and HindIII and the isolated 3' fragment from pPAL896 was cloned into these sites, reconstructing a promoterless version of the gene that contains the indicated array of restriction sites flanking the gene. This plasmid was named pPAL895 35 and is shown in Figure 15.

Examples 36

This example describes the isolation and construction of a promoterless version of the gene 1, IamS: indole acetamide synthase gene of the Ti plasmid of the Agrobacterium tumefaciens strain C58 which procedure is summarized in Figure 16. The gene was isolated from the plasmid pPCV311. The SmaI - PstI fragment that contains 5' and 3' regions of the IamS gene as well as the coding region was isolated by gel elution and subcloned into a derivative of pGEM 4Z called pGEM-noEco. pGEM-noEco is a plasmid from which the EcoRI site of pGEM 4Z has been removed by cutting with EcoRI and making blunt ended and relegating such that only the EcoRI site was removed. The fragment was inserted in the orientation shown relative to the polylinker. This subclone was called pPAL889. pPAL889 was digested with EcoRI, and briefly treated with Exonuclease III, followed by S1 nuclease. The DNA was digested with SmaI and treated with Klenow fragment to make it blunt ended. The DNA was relegated and clones recovered. Some of these clones were chosen, sequenced, and one clone was found which had 5' sequences deleted such that only approximate 15 bases upstream of the ATG start of translation codon remained. This plasmid was named pPAL888. The KpnI site at the 5' end of the gene as well as the PstI site at the 3' end of the gene were both converted to SalI sites by cutting with KpnI, end filling with Klenow and adding synthetic SalI linkers, and repeating the linker addition at the PstI site such that the entire gene can be excised as a single SalI fragment. This plasmid was named pPAL887. This plasmid contains the promoterless version of the IamS gene and contains the array of restriction sites shown that flank the gene as shown in Figure 16.

Example 37

This example relates to the detailed characterization of the coding regions of clone number L4 a microspore specific clone isolated from a Brassica napus genomic library and the construction of vectors containing these

genes. Figure 2a is a schematic representation of the restriction map and coding regions of clone number L4. The clone contains three different members of the same gene family. These genes are identified as Bp4A, Bp4B and Bp4C. The first (Bp4A) and third (Bp4C) genes are functional, the second gene has modifications that most likely render it non-functional. The restriction map is made diagrammatically in that the non-transcribed regions are shown as a single line, while the transcribed regions are shown as a boxed area. The second gene (Bp4B) is identified on the basis of sequence homology and is therefore boxed with a dotted line. The notation "del 220" refers to an approximately 220 base pair deletion/rearrangement affecting the second gene (Bp4B) in this clone. Start of transcription is located at the leftmost side of each boxed area (except in the case of gene Bp4B) and exon and intron positions are noted by the exons being filled in with black and the intron positions being left unfilled. A small arrowhead is shown on the non-transcribed 5' region of each gene, this arrowhead serves to indicate the promoter region of each gene. Restriction sites are identified such that the number of the first nucleotide of the restriction enzyme recognition site is shown. Not all restriction sites are shown, only those relative to the constructs detailed within are indicated. The genes are presented with the 5' region being on the left side and the 3' region being on the right. The numbering of the DNA sequence in all cases starts from left to right, 5' to 3'.

In Figure 3a, the DNA sequence of the clone L4 is shown, the orientation of the sequence and the genes contained within the microspore specific clone from *Brassica napus* are from 5' to 3'. In Figure 3a, clone L4, nucleotide 1 in the complete sequence is at the left-most EcoRI site while nucleotide 8579 is at the first nucleotide of the right-most EcoRI site. The start of transcription of gene 1 in clone L4 is nucleotide 235. The 5' and 3' intron splice sites are identified in

boldface type. The start ATG codon is shown as well as the stop termination codon. The deduced amino acid sequence of the proteins encoded for by these genes are also shown. The end of transcription for gene 1 is approximately nucleotide 1427. As indicated above the second gene in clone L4 is most likely non-functional due to an insertion and a deletion that occurs in the region of the promoter and first exon. This gene was not utilized for constructs. The third gene in clone L4 has a transcriptional start at position number 6298 in the DNA sequence and transcription ends at approximately nucleotide 7490. The ATG start codon, intron splice sites and termination stop codon are all identified as above. Vectors were constructed from this clone by using promoter fragments from both genes 1 and 2. The specific promoter fragment constructs are detailed below.

The construction of 2 vectors containing promoter and promoter fragments from the clone L4 was carried out as follows and as shown in Figure 7 (A,B,C,D). The first vector was constructed by first subcloning the EcoRI-SstI (nucl. 1-2132) fragment containing the first gene of clone L4 (235 base pairs of promoter/exon/intron/ second exon) in the commercially available vector pGEM-4Z (Promega Biotech, Madison, WI, U.S.A.) using the EcoRI-SstI sites of the polylinker of this vector. This plasmid was named pPAL0402. The 2.7 Kb EcoRI fragment of clone L4 that contains the third gene (Bp4C) was then cloned into the EcoRI site of pGEM 4Z, leading to a plasmid called pPAL0411. The plasmid pPAL0402 was then digested with EcoRI and the 2.7 Kb EcoRI fragment from pPAL0411 (nucl. 5859-8579) that contains the gene number three (Bp4C) from clone L4 was added to it. Clones were recovered that contained this inserted 2.7 Kb EcoRI fragment in both orientations relative to the promoter region of the first gene. A clone that contained this third gene fragment in an orientation such that the promoter from the third gene was opposite to the promoter in the first gene was chosen and called pPAL0403.

pPAL0403 contains the entire third gene from clone L4 oriented in such a fashion as to have the promoter region immediately adjacent to the 235 base pair promoter region of the first gene in pPAL0403. This plasmid, pPAL0403
5 was digested with DdeI. DdeI digestion produced a fragment of approximately 1.9 Kb. The DdeI sites are located at nucleotides 303 and 7366. Because of the orientation of these fragments, digestion with DdeI produces a 1.9 Kb fragment. This 1.9 Kb fragment
10 contains a copy of the third gene (Bp4C) oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 (Bp4A) which is transcribed from left to right, ending in a DdeI site
15 that is located 67 basepairs down stream of the major start site of transcription and proceeds the ATG start of translation codon by 2 nucleotides. This 1.9 Kb DdeI fragment made blunt with Klenow fragment and cloned into the XbaI site of the polylinker region of pGEM 4Z
20 previously made blunt ended with Klenow fragment. The resultant plasmid pPAL0408, was recovered and subsequently was digested with SalI and SstI, which releases the cloned DdeI fragment bordered by on the left hand side, (nucl 7366) SalI and on the right hand side
25 (nucl 303) of this construct and contains a portion of the polylinker of pGEM 4Z containing the following unique sites: BamHI, SmaI, KpnI, and SstI restriction enzyme sites. This SalI - SstI fragment was cloned into the SalI - SstI sites of PAL1001. PAL1001 is the binary
30 vector Bin19 (described by Bevan, M., Nucleic Acids Res., 1984, 12:8711-8721) to which has been added the nor ter polyadenylation signal as a 260 bp SstI - EcoRI fragment isolated from the plasmid pRAJ221 (available from Clonetech Laboratories, Palo Alto, CA, U.S.A.) in the
35 SstI - EcoRI sites of the polylinker region of Bin19. The nos ter is identified as a stippled box. The binary transformation vector that resulted from the insertion of the SalI - SstI fragment of pPAL0408 into PAL1001 was

named PAL1107. The details of the construction are shown in Figure 7A. This vector has a copy of the third gene oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 which is transcribed from left to right, followed by a polylinker with unique sites for the insertion of DNA which consist of: BamHI, SmaI, KpnI and SstI followed by the nos ter. This vector has the feature in that additional 5' non-coding sequences were placed upstream to the 235 base pair core promoter on Bp4A, but these additional 5' sequences were in an opposite orientation. The provision of these sequences in this orientation does not affect the pollen specificity of the core 235 base pair promoter. With this vector, one can also ascertain for position effects of the transformation process in that since the vector contains an intact copy of the Bp4C gene, probing for the levels of expression of the Bp4C gene can give an indication as to what levels of expression from the other promoters essential for pollen development and/or function in PAL1107 can be expected.

Example 38

This example describes the construction of additional vectors having the promoter regions of the genes contained in clone L4 which may be useful for pollen specific expression of gene sequences. The three genes within clone L4 (Bp4A, Bp4B, Bp4C) show very near-exact DNA homology and this is most apparent between the first (Bp4A) and third (Bp4C) gene. The second gene (Bp4B) is a homologous copy that has undergone sequence changes that have appear to have lead to inactivation of it. The extensive similarity between the first, second and third genes in clone L4 is also maintained in the promoter region such that out of the first 235 nucleotides of the first and third gene promoter regions there are only 5 nucleotides that differ between them. Downstream of the TATA box in these two promoters the only difference between them is the presence of one additional nucleotide

- 130 -

at the start of transcription (e.g., Promoter 1, Bp4A:
.....TATGTTTtAAAA... versus promoter 3, Bp4c:
.....TATGTTTAAAA (transcribed region underlined,
single nucleotide difference shown in lower case).

5 However, within the sequence of the first gene there is
a nucleotide change that introduces a DdeI site (nucl
303) in the untranslated 5' leader sequence upstream of
the ATG start codon that is not present in the
untranscribed leader sequence of the third gene in clone
10 L4. For this reason, chimeric promoter constructs were
made which utilized this DdeI site in the first gene
joined to sequences from the third gene promoter. The
region of the first promoter used for these constructs
consisted of the sequences contained between the SnaBI
15 site (nucl 210) near the TATA box to the DdeI site
located immediately upstream of the ATG start codon in
the first gene (nucleotide 303 is the first nucleotide in
the recognition sequence for DdeI). The other region of
this chimeric promoter (5' of the TATA box) was a
20 fragment extending from the EcoRI site of the third
promoter (nucleotide 5858) to the SnaBI site near the
TATA box (nucleotide 6273). Therefore to facilitate
construction of these pollen specific vectors, the
following reconstructions were performed.

25 The EcoRI to DdeI fragment that encompasses the
promoter region of the first gene in clone L4 was
isolated by first cutting pPAL0402 with DdeI, blunting
with Klenow, and then cutting with EcoRI. The 235 base
pair fragment corresponding to this region was cloned
30 into the EcoRI - SmaI sites of pGEM 4Z. This plasmid
(pPAL0422), was then cut with EcoRI and SnaBI. A DNA
fragment that contained the EcoRI to SnaBI portion of the
promoter for gene 3 in clone L4 was isolated by digesting
pPAL0411 with EcoRI and SnaBI. This released an
35 approximately 415 base pair EcoRI (nucl 5858) to SnaBI
(nucl 6273) fragment that represents most of the 5'
region of the gene 3' promoter from clone L4 (the SnaBI
recognition site is 2 base pairs downstream of the TATA

box). This EcoRI - SnaBI fragment was used to replace the shorter EcoRI - SnaBI fragment removed from the first promoter subclone (pPAL0422), reconstructing a promoter fragment of approximately 550 base pairs. This plasmid is referred to as pPAL0421. This chimeric promoter fragment contains 415 base pairs of the promoter of gene three in clone L4, followed by approximately 99 nucleotides of the first gene promoter/untranslated leader sequence.

For construction of a pollen specific cassette vector, the following plasmids were first constructed. The first plasmid constructed contained the nos ter polyadenylation signal with a polylinker in front of the nos ter. This was accomplished by first isolating from pRAJ221 the nos ter as a SstI - EcoRI fragment and this fragment was cloned in pGEM 4Z using the SstI and EcoRI sites in the polylinker. This subclone is referred to as pPAL001. To pPAL001, a fragment coding for neomycin phosphotransferase (NPT II) derived from the plasmid pRAJ162 was added to it in the antisense orientation as follows: The plasmid pRAJ162 contains the NPT II gene from the transposon TN5 inserted as a SalI fragment and bounded by a polylinker in the plasmid pUC-9 and was obtained from the Plant Breeding Institute, Cambridge, U.K. pRAJ162 was digested with HindIII and SmaI. The DNA fragment containing the NPT II gene was isolated by elution from an agarose gel. pPAL001 was digested with HindIII and SmaI and the NPT II gene fragment was ligated into it. The resultant plasmid was called pPAL002 and had such orientation of restriction sites and the NPT II gene and nos ter as follows: HindIII, PstI, SalI, 3' end NPT II coding sequence 5' end, SalI, BamHI, SmaI, KpnI, SstI, nos ter, EcoRI. pPAL002 was cut with HindIII and the site made blunt ended by the use of Klenow fragment. pPAL0421 was digested with HincII and pVUII, both of which leave blunt ends, and the promoter fragment was ligated into HindIII cut blunt ended pPAL002. Plasmids were obtained that contained the promoter in both

orientations relative to the nos ter. One plasmid was chosen with the proper orientation (5' promoter/antisense NPT II/nos ter) and was named pPAL0419. pPAL0419 has the following DNA fragments: A small (approx. 130 bp) of pGEM 4Z that contains the SP6 promoter, the 550 base pair chimeric promoter, the NPT II gene in the antisense orientation relative to the promoter, followed by the nos ter polyadenylation signal. This entire promoter/NPT II/nos ter construct is excisable by EcoRI. In order to provide promoter sequences that could be utilized with additional gene constructs, the plasmid pPAL0419 was digested with SalI. This digest removes the NPT II coding region and this SalI digested pPAL0419 was relegated giving rise to pPAL0402. pPAL0420 represents the promoter essential for pollen development and/or function followed by a polylinker for insertion of genes that has the following unique sites: HincII, PstI, SalI, BamHI, SmaI, KpnI, SstI, followed by the nos ter polyadenylation signal. The entire promoter/polylinker/nos ter construct can be conveniently excised as a single EcoRI fragment. The details of this construct is shown in Figure 7B. This plasmid was used for the construction of an additional promoter essential for pollen development and/or function in a binary transformation vector. The intact L4 clone in the lambda cloning vector was digested to completion with the restriction enzymes SstI and HhaI. The resultant fragments were separated by gel electrophoresis and a 2.65 Kb fragment that contains the promoter/first exon/intron/partial second exon region of gene three in clone L4 and corresponds to nucleotides 4565 to 7216 in the sequence of clone L4 was isolated. This fragment was made blunt ended with Klenow and cloned into the binary transformation vector PAL1001 previously described with reference to Figure 7a. PAL1001 was first cut with HindIII and made blunt ended with Klenow. Clones containing this fragment (promoter/first exon/intron/partial second exon) were recovered. A clone

was chosen that contained this fragment in the proper orientation such that the direction of transcription was towards the *nos ter* in PAL1001. This vector was named PAL1421. This vector contains approximately 1.9 kb of upstream promoter region from the gene 3 in clone L4 followed by the first exon, the complete intron and 15 bases of the second exon of gene three followed by a polylinker containing the following unique sites: *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sst*I, and finally the *nos ter* polyadenylation signal. A variant of this vector was constructed by digesting PAL1421 with *Eco*RI and replacing the promoter/exon/intron/second exon/polylinker/*nos ter* structure with the promoter/polylinker/*nos ter* structure from pPAL0420 using *Eco*RI such that a longer 5' promoter region is reconstructed in the binary transformation vector. The resultant vector was named PAL1423. The outline of this construction is shown in Figure 7D.

Example 39

In this example, a promoter essential for pollen development and/or function is used to synthesize the enzyme *IamH* specifically in pollen cells. The enzyme has activity that can cause the production of NAA from NAM, the substance NAA functioning as a plant hormone that is substantially toxic to developing pollen grains, while the precursor NAM being relatively non-toxic. For this example, the *IamH* gene was inserted into the vector PAL1423. The *IamH* gene was isolated from pPCV311 as described in Figure 15 and cloned as a *Sal*I fragment in the *Sal*I site of PAL1423, creating PAL1426. This vector has the *IamH* gene (T-DNA gene 2) under the control of a promoter essential for pollen development and/or function from clone L4 in the sense orientation. PAL1426 was used to transform tobacco as outlined in Example 34.

Example 39a

In this example, constructs are made whereby the *IamS* (gene 1) or *IamH* (gene 2) are expressed from either the microspore-specific promoter *Bp10* or the anther-specific *TA39*. The four constructs are shown in Figure 23

(Plasmid pPHP5838, Bp10:IamS), Figure 24 (pPHP5839, Bp10:IamH), Figure 25 (pPHP5840, TA39:IamS), and Figure 26 (pPHP5841, TA39:IamH). All four constructs are generated using as a backbone the plant transformation vector pALLTKREP (Boutilier, K.A., Gines, M.-J., DeMoor, J.M., Huang, B., Baszczyński, C.L., Iyer, V.N., and Miki, B.L. Expression of the BnmNAP subfamily of napin genes coincides with the induction of Brassica microspore embryogenesis. 1994. Plant Molec. Biol. 26: 1711-1723). pALLTKREP was renamed at Pioneer and will be referred to elsewhere as PPHP1741.

The relevant portions of pPHP1741 that are retained in the four Pioneer plasmids include: (1) a wide host range bacterial vector, including a bacterially expressed kanamycin resistance gene as the selectable marker, (2) left and right T-DNA borders derived from *Agrobacterium tumefaciens*, and (3) between the T-DNA borders, a chimeric selectable marker gene for plant expression of resistance to kanamycin, comprising a CaMV 35S promoter operably linked to the NPTII open reading frame (ORF) and CaMV 19S 3' nontranslated region, which encodes signals required for proper processing of the 3' end of the transcript. Note in the maps, the wide host range origins of replication are referred to as "OriV" and "OriT" (vegetative origin of replication and transfer origin of replication); the bacterial selectable marker is referred to as "S. F. KanR"; the T-DNA left and right borders are referred to as "LB" and "RB", respectively; the CaMV 35S promoter is referred to as "35SPro"; and the CaMV 19S 3' region is referred to as "CT".

In Figures 25 and 26, incorporated between the TA39 promoter and the IAMS or IAMH genes is the nontranslated leader from Tobacco Mosaic Virus (TMV), 0', (Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C., and Wilson, T.M.A. 1987. The 5'-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucl. Acids Res. 15: 3257-3273; Gallie, D.R., Sleat, D.E., Watts, J.W.,

Turner, P.C., and Wilson, T.M.A. 1987. A comparison of eukaryotic viral 5'-leader sequences as enhancers of mRNA expression in vivo. Nucl. Acids Res. 15: 8693-8711; Sleat, D.E., Hull, R., Turner, P.C., and Wilson, T.M.A. 1988. Studies on the mechanism of translational enhancement by the 5'-leader sequence of tobacco mosaic virus RNA. Eur. J. Biochem. 175: 75-86). 0' is our designation on the maps of Figures 25 and 26 for the TMV non translated leader, which is known as "0'".

10 The constructs also contain the 3' nontranslated region from either the nopaline synthase (nos) gene from Agrobacterium tumefaciens Ti plasmid pTiT37, or from the proteinase inhibitor II gene (PI-II) from Solanum tuberosum (potato). Both are well-known in the art. The reference for the nos 3' region is: Bevan, M., Barnes, W.M., and Chilton, M.-D. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. Nucl. Acids Res. 11: 369-385. The reference for the PI-II 3' region is: An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R.W., and Ryan, C.A. 1989. Functional Analysis of the 3' Control Region of the Potato Wound-Inducible Proteinase Inhibitor II Gene. Plant Cell 1: 115-122. Note that in Figures 23-26, the nos 3' region is referred to as "NT" and the PI-II 3' region is referred to as "PinII".

25 Figures 27-30 list partial sequence information for plasmids pPHP5838, pPHP5839, pPHP5840 and pPHP5841 for which maps are provided in Figures 23-26, respectively. The nucleotide numbers indicated in Figures 27-30 are the same as the numbers on the maps of Figures 23-26. The sequences provided in Figures 27-30, extend from the left border region to the right border region of each plasmid. That part of the DNA sequence not included is common to all four plasmids and belongs to plasmid pPHP1741 (pALLTKREP, described above).

30

35

Example 40

The vector PAL1426 was used to transform *Brassica napus* as described in Example 34.

Example 41

5 In this example, the vector PAL1107 was used for the
production of tissue-specific GUS (beta-glucuronidase)
enzyme. The gene for this enzyme is available from
Clontech Laboratories, Palo Alto, CA., U.S.A. The gene
was inserted to PAL1107 as a BamHI - SstI fragment and
10 was used to transform tobacco as described in Example 34.
Plants produced had detectable GUS activity only in
developing pollen cells, and not any other tissue tested.
The application of a non-toxic analog of glucuronic acid
to which has been conjugated a toxic molecule could be
15 applied to these plants and cleavage of the toxic moiety
from the glucuronic acid would occur only in pollen
cells. This provides an example of an enzyme that could
be used for the production, in a tissue-specific fashion,
of a toxic substance from a non-toxic analog. One such
20 analog that could be used is Gluc-Camp, a glucuronic acid
analog that is conjugated to chloramphenicol. When acted
upon by glucuronidase, chloramphenicol is produced,
inhibiting cellular growth and development.

Example 42

25 In this example, two isogenic plant lines (A1, A2)
were produced that carried either the IamS or the IamH
genes. Tobacco plants were transformed with PAL1426
containing the IamH gene as in Example 39, producing the
A2 line. The IamS gene described in Figure 16 was
30 inserted as a SalI fragment into the vector PAL1423 in
the sense orientation, giving rise to PAL1425. PAL1425
was used to transform tobacco as described and tobacco
plants were produced that carried PAL1425. These lines
represented the A1 lines. Tobacco plants that contained
35 both PAL1426 and PAL1425 were selfed and homozygous A1
and A2 lines were selected.

Example 43

In this example, PAL1426 (see Example 39) and PAL1425 were used to transform *Brassica napus*. Plants lines homozygous for the A1 and A2 genes were selected as in
5 Example 42.

Example 44

Gene 1 (IamS) and gene 2 (IamH) constructs were introduced into different canola lines by transformation, as in example 34. Two different anther-specific
10 promoters were used for each gene: TA39 from tobacco and Bp10 from *Brassica* constructed as described in example 39a and Figures 23-26. T0 plants carrying each gene/promoter combination were recovered. The T0 plants carrying gene 2 looked completely normal, and were
15 completely male and female fertile. Some of the T0's carrying gene 1 were abnormal, showing huge flowers and not much pollen. The pollen from these plants was probably not fertile. In the crosses described above, the more normal looking gene 1 plants were generally
20 used. Crosses were made between plants containing gene 1 and gene 2. Eight T1 progeny which are PCR-positive for the presence of both gene 1 and gene 2 were identified. The following T1 combinations were obtained:
25 TA39/gene 1 + TA39/gene 2 (2 plants); TA39/gene 1 + TA39/gene 2; TA39/gene 1 + TA39/gene 2; BP10/gene 1 + TA39/gene 2; BP10/gene 1 + TA39/gene 2 (2 plants); BP10/gene 1 + BP10/gene 2 (2 plants); BP10/gene 1 + BP10/gene 2. The T1 progeny were male sterile and female fertile. The male sterile plants
30 showed very reduced anthers and either lacked pollen or the pollen was brown colored. In some cases, the male sterile plants actually produced pods, but no seed was present. One of the T1 progenies only, when selfed, produced a limited amount of seed. Female fertility was
35 confirmed by applying wild-type pollen on the plants which resulted in seed production.

It Is Claimed:

1. A recombinant DNA molecule for use in preparing a male sterile plant of a pollen-bearing species that is capable of being transformed, said molecule comprising
 - 5 (A) a first DNA sequence coding for a first gene product that converts a first substance which is present in the plant into a second substance, wherein the first and second substances do not inhibit pollen formation or function,
 - 10 (B) a second DNA sequence coding for a second gene product that converts said second substance to a third product that inhibits pollen formation or function,
 - 15 (C) a first promoter that controls expression of said first DNA sequence in plants,
 - (D) a second promoter that controls expression of said second DNA sequence in plants, and
 - (E) a third DNA sequence that confers resistance to a selective agent.
- 20 2. A recombinant DNA molecule according to claim 1, wherein said resistance is to a herbicide.
3. A recombinant DNA molecule according to claim 2, wherein said herbicide is selected from the group consisting of glyphosate, glyphosinate and TBL.
- 25 4. A recombinant DNA molecule according to claim 1, wherein at least one of said first and second promoters is specific to cells that are critical to pollen formation or function.
- 30 5. A recombinant DNA molecule according to claim 4, wherein at least one of said first and second promoters is specific to anther cells.
6. A recombinant DNA molecule according to claim 4, wherein at least one of said first and second promoters is specific to microspores.
- 35 7. A recombinant DNA molecule according to claim 1, wherein said second promoter is an inducible promoter.

8. A recombinant DNA molecule according to claim 1, wherein said first DNA sequence is IamS and said second DNA sequence is IamH.

5 9. A recombinant DNA molecule according to claim 1, wherein said second substance is 2-amino-4-methoxybutanoic acid and said second expression product is methoxinine dehydrogenase or rhizobitoxine synthase.

10 10. A recombinant DNA molecule according to claim 1, wherein said second substance is a non-toxic analog of glucuronic acid and said second expression product is β -glucuronidase.

15 11. A recombinant DNA molecule according to claim 1, wherein said second substance is naphthalene acetamide or indole acetamide and said second expression product is indole acetamide hydrolase.

12. A method of producing a male sterile plant, comprising the steps of

20 (A) introducing a recombinant molecule according to claim 1 into the genome of a pollen producing plant which is capable of being genetically transformed; and

25 (B) growing said pollen producing plant under conditions such that male sterility is achieved as a result of the expression of said first DNA sequence and said second DNA sequence, providing said male sterile plant.

30 13. A method according to claim 12, further comprising, after step (B), the steps of (C) crossing said male sterile plant with an fertile, isogenic plant to obtain seed, then (D) growing out said seed to produce progeny plants, and (E) selecting male sterile plants from among said progeny by exposure to said selective agent.

35 14. A method of producing hybrid seed from a male sterile plant, comprising the steps of

(A) introducing a first recombinant molecule according to claim 1 into the genome of a pollen producing

plant which is capable of being genetically transformed;

(B) growing said pollen producing plant under conditions such that male sterility is achieved as a result of the expression of said first DNA sequence and said second DNA sequence, yielding a male sterile plant; and

(C) crossing said male sterile plant with pollen from a male fertile line, said pollen having integrated into its genome a second recombinant DNA molecule comprising a selective marker gene and a promoter regulating expression of said selective marker gene in plants.

15. A method according to claim 14, wherein said second recombinant DNA molecule further comprises (i) a third DNA sequence and (ii) a third promoter that causes transcription of said third DNA sequence in plants, wherein said third DNA sequence is transcribed as an antisense RNA or encodes a gene product that negates the effect of said first DNA sequence or said second DNA sequence, such that hybrid seed with restored fertility is produced by said male sterile plant, and said third promoter causes said transcription such that expression of said third DNA sequence overlaps in time with expression of said first DNA sequence or said second DNA sequence.

16. A method according to claim 15, wherein said third DNA sequence has an antisense RNA transcript.

17. A method according to claim 15, wherein said third DNA sequence is a repressor gene.

18. A method according to claim 15, wherein said third DNA sequence encodes a gene product that is a ribozyme.

19. A method according to claim 15, wherein said selective marker genes imparts herbicide resistance.

20. A method according to claim 15, wherein said third DNA sequence encodes a gene product that is a dominant negative transactivator.

21. A method according to claim 15, wherein said third DNA sequence encodes a gene product that is a transdominator.

22. Hybrid seed produced by a method according to
5 claim 15.

23. A plant cell comprising elements (A) through (E) of a DNA molecule according to claim 1.

24. A differentiated plant comprising a cell according to claim 23.

FIG. 1

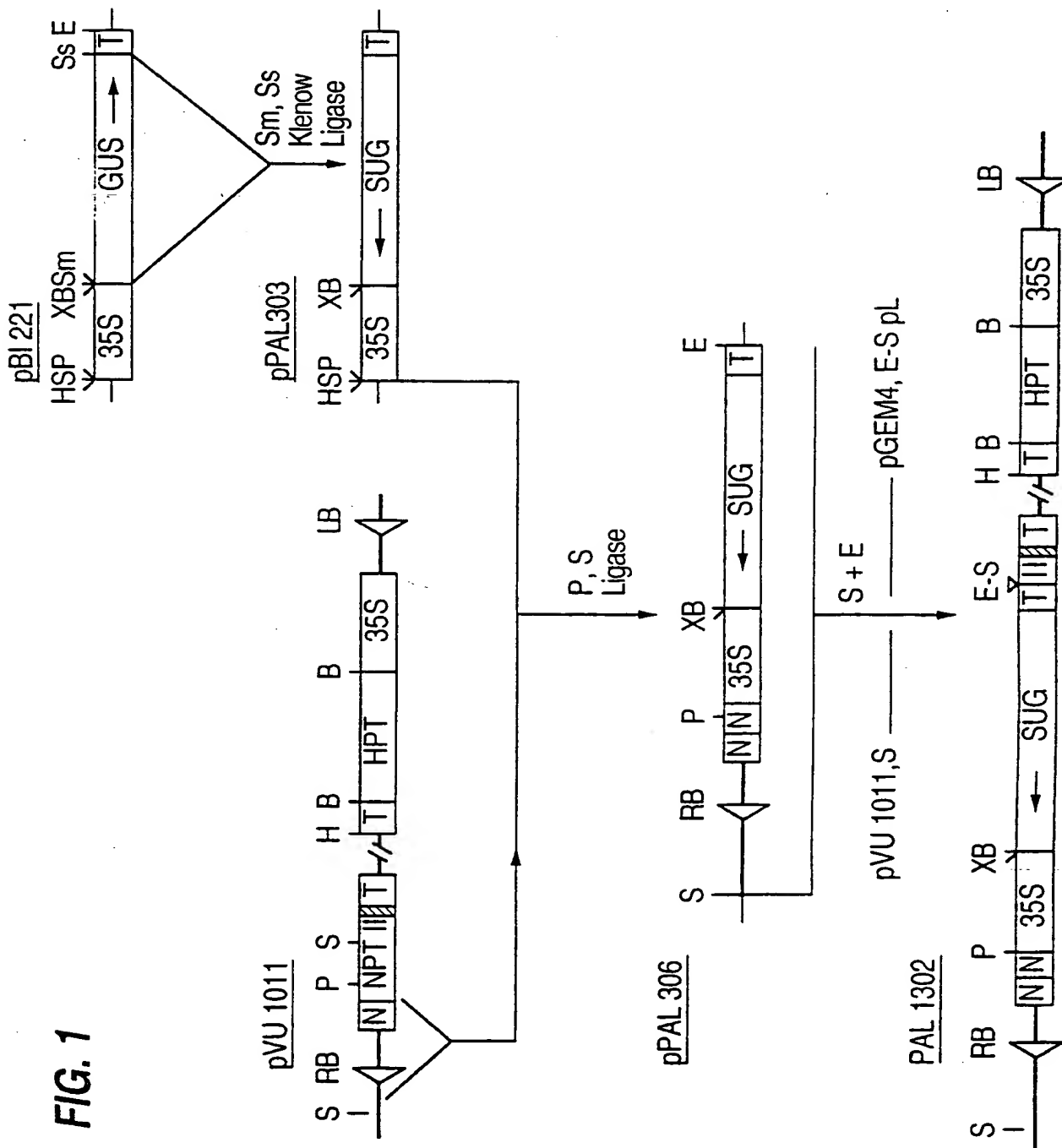


FIG. 2a

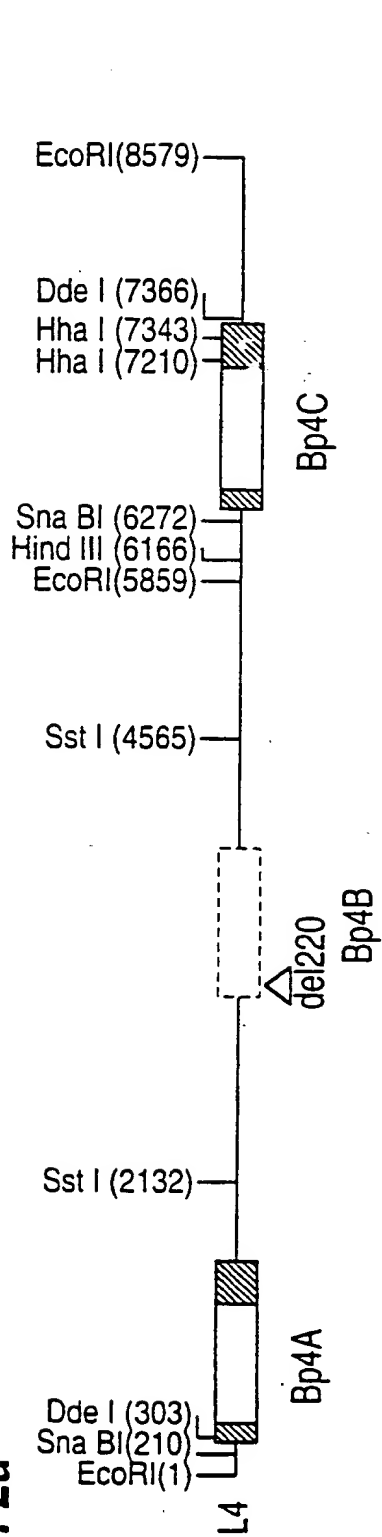
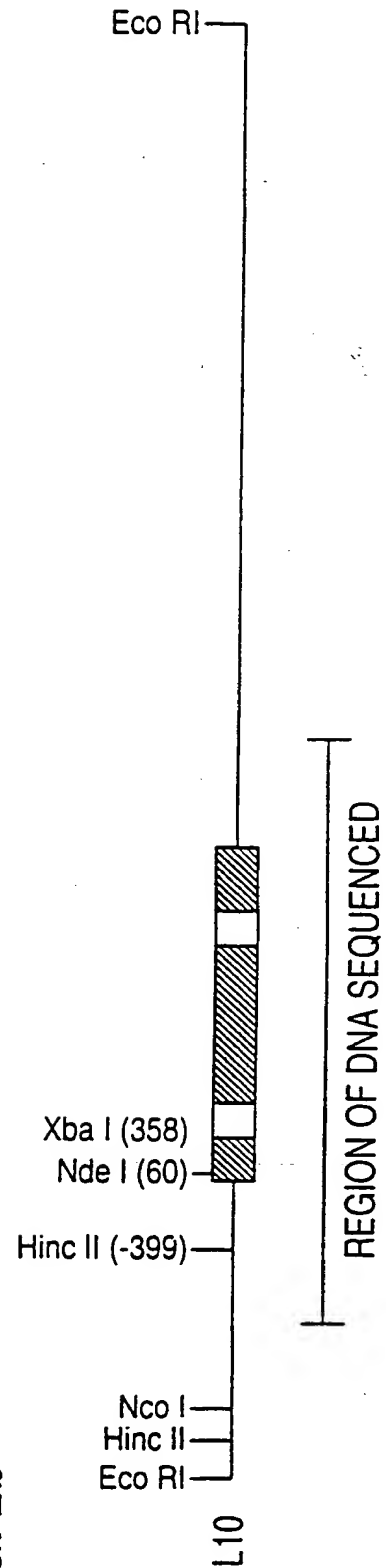
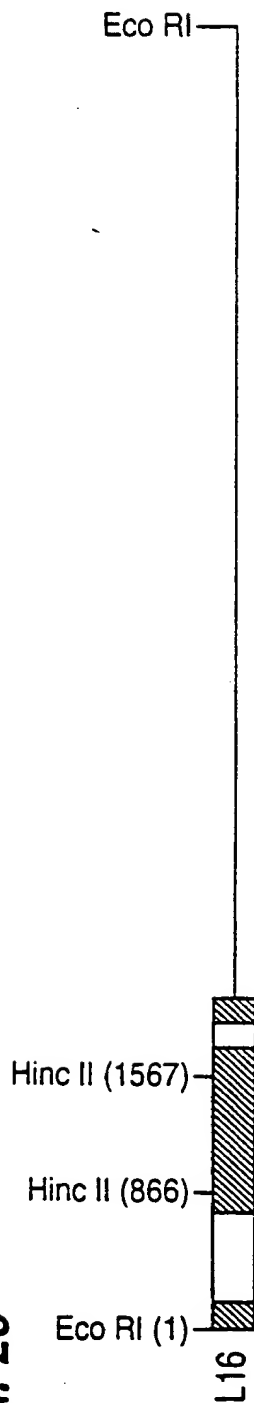


FIG. 2b



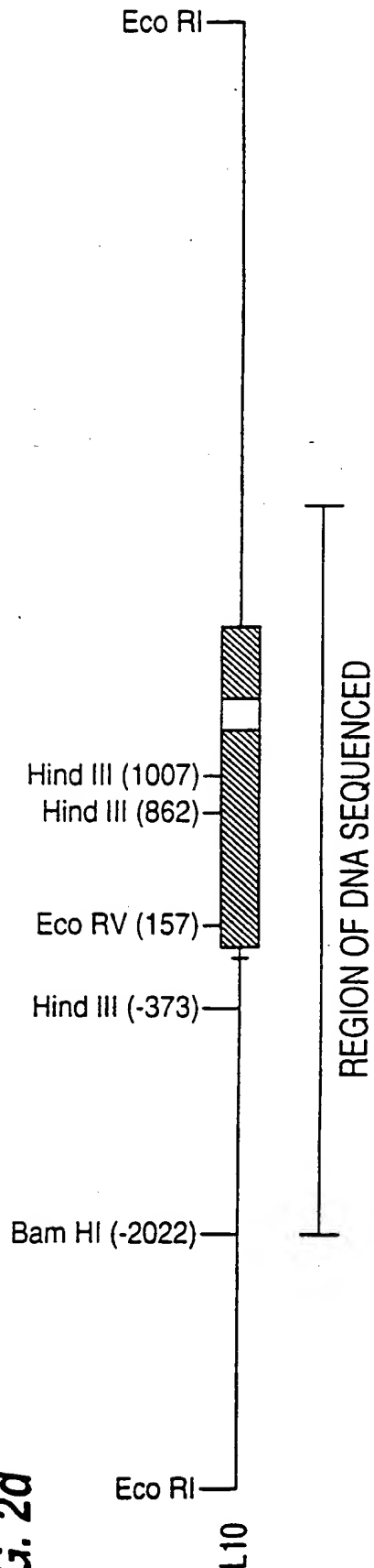
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FIG. 2c



REGION OF DNA SEQUENCED

FIG. 2d



REGION OF DNA SEQUENCED

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FIG. 3a(1)

1 GAATTCATAAAATAGCAATAACTTTTIGAGAACATCAGATTTATGTACACGCATAGGACA 60
61 CATACCTTTTTTATTTACTTTAAAGGAAATCAACGAGTCTAAATCTTCCACATGTTATATG 120
121 AGCAAAACATGAATTTTTTCTAAATTAGAATTCGTTTAAATCAGAACATATTATGTGAGTT 180
181 TCTTAAATTAGATTTTTTAATATCTATATATAGTAAGTAATACITCTTATGTTTFAAAATA 240
241 AAAAATAGATACTTTCATCTCTTTCCTAAATTTTTTAAGCCATATCAATCCATTTTCTATA 300
301 ATCTAAGATGAAGAAATCCCTTCAACTCTCTTTTTCGTTCTTAAATATCTCCATCTCT
M K K S L Q L S F S F L I I S I I L
361 CTCACATGGTTTIGTATTTTTCATCTTAAATATATTTGCCATATAGTAATTCATTAATAATTGA 420
S H G
421 TTATACTATAAAATTTGACITTTTAAATATTGTGCAACCCCATATAATAAATTTTATTTACT 480
481 ATATAAAACATAGCCATTAATTTATCTCTTTTGTGTAAATTTTCATAACTTTGCCAGAAGGCTA 540
541 GAAATATATAGTAGTATAGTCAGAAATGTTTTCGGTTAAATTTGAAAGGATCAACCATGGA 600
601 GTATTTAAATGTTTTTTATACITTTTATGCCATTTTATAATTTTTTTTAAATGTATCGGGTTATA 660
661 TATGATCAAGAACTATTATGATAAAATAATAATAAATTTTCAATTTTATCATCTATTT 720

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FIG. 3a(2)

721	ATGAACAATTTTGTCTCTGCACATACAAATGATTTTAACCAACAATTTTAAATAATATGGATGA	780
781	ACTATAGCTCTTACGTAAATTTATTTTGATATTTTAAATTAAATTTATATATATTTTATATAGG	840
841	TAATTTGTATTGCTTTTCCCAATACATACAGTAGTTGTTTATTTAAATATCAAAATTTAATA	900
901	CGTAATGTTTATTAATATGCCACACAATTCITTAATAACCATATTTTFAAAAAATAATGIGIG	960
961	ACCAACGATATGCTCATTTTATTTTATTACTAGCAAAATATATTTCTTTTCTTACTTTATA	1020
1021	ACGTTTAAAAGAAATGTTATTAACATTTTTCGCTGATAAATAAATTTTATATTTTTCATA	1080
1081	AAATCTAAATATATTTTAAACAATTAATAATTTTGAATAATTTTATATCTTTACAGGAATGAT	1140
	M M	
1141	GGCAGATGCCGAGAAAAGAAATTTGCTCTCATATAAATTCCTCAATAAAGGAAGTTATTTGTC	1200
	A D A Q K K N C P H K I P I K G S Y C A	
1201	TCCAACATATATGTTTGGATATGTGTAGAAGCAACATGGAACCTGTTGGTAGTTGTCGCGGA	1260
	P T I C L D M C K K Q H G T V G S C A E	
1261	ATAAAAATGATTTTGTAACTCGCTTTGTAAGTAAGGGTTCTCACTAAGTGTATGAATCT	1320
	STOP	

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FIG. 3a(3)

1321 AGTAATGICCAACCAAGTTTATATTTATTTCTTTTAAACAATPAAGTCTFAAATGTTGTCT 1380
1381 CAGATTGIGGATCTATTATTAATAAATAATATATGAATGTTAAATTAATACAATGIG 1440
1441 TAAAPACAAGAGTGGACTATTAAATAAATAATATATGATTACATTTATTTGTTAGAAGTAAACCAAT 1500
1501 ATTACGIGTAAATAATCAAAATCTTAAGACAAGTTTAAAAAAGATTGAGATGAATAATCACAAACCA 1560
1561 ATATTTAAATGIGAGATAATCAACTTAACATGTATTTTGTACACATTTGTAAAAAATAAAAA 1620
1621 AGCAAGAGTTCAATATCAACAAGAAAGTTTAGAAAAGAGCAACAGATTTCATTCGAAGGG 1680
1681 CAGICTAGGTTGAATTGGCTTGACATAGGGAATAATTTGAAAGCACACTGTTTCTTGAACATGAC 1740
1741 AACGCTTGGTCAGGAAGAAACAATCTCAACCAAGAGTTTGGGTAGATTTCCTCCATGTC 1800
1801 ATTATCAGGTACGAGTTATGAGACTTTCATCCACATCTCAGTCCAGTTCCCTTCTCAGGA 1860
1861 AGTTTCCTTGAGGAAGGAGGTTATTACAGAAAGCTPAAGTTACATGAGCCTGACATATCAT 1920
1921 GCAAGGCCAGTCCCAACAAGAAAATGTTAGAAAGAGCAACATATCATCCAGGACAGTCC 1980
1981 AGGTTTGAATTGGCTTGACAGATGGTTTGACAGACATGCCATCTGTAAGGTCCTACAACATC 2040
2041 ATCAGACAAACGAAGGAAAATTGATAGCAATTGTTCTGAAACATGACAAAACCTCTAGTCAAG 2100

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FIG. 3a(4)

2101	AAGAAGAATCTCAACCAAGTTTGGGTAGAGCTCCTCCAATGICATCATCAGCTACG	2160
2161	AGTTCAAGAGACTTCACATCTTAGTCCCATCTCTCTCTCATGAAGTTTCCTTTAGG	2220
2221	AAAGAGGTTATTACAGAAAGCCAAAGTTACATGAGCCTAACACAATCTATCAAAGCTAAGA	2280
2281	AGAGACGATTAAACCATTTATTTCTTCATCTTTTGTCCCAAAATCACCTGTTTGAAAAAGAAAC	2340
2341	AGTCCATGTCTTACAAACGAAGATGIGAATGTAAAGCGTTGTGTCATGTTCCGATCCATCTT	2400
2401	GCAACAGCAGAGTTATCTATATATCCACATGCTCAAGTAAACAAGGAAAAATATGTGGGCAA	2460
2461	AGAAAGGTAAAGTGAACAATCGTTTCATTAATTCATTTAAAGCATTTCAACACCTTGATGGTT	2520
2521	CTAATATACACACAAAACACTGATTTATATAGATACATAAGCAACTTTCGTGTATGTTCTTTT	2580
2581	ACATACAAGTTGGTATCAAGGCCTGTGCAATGTTGTGTTGTGACCACTTTTATTTATTTAA	2640
2641	TAGTTAACTTTTGTATGCTTCTPAAGATAATATGTTTCCTCCTAACTCTTGICACACATGAAG	2700
2701	ACCAATTAAAGGTTTGTATTAATAATACATPACTAAATTTTAAATAATAATCTTATAAGTTATG	2760
2761	TTACGTGTGGGACATCCACCTTAATAAACTATAAATTTAAATAATAATGTTTGAAAAAGGAT	2820

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FIG. 3a(5)

2821 TTTATTGACATTCCTTTAAATAAATTCATATAATTTTAAAAATPAGCGATAACTTTTGTGAAA 2880
2881 CATCAGAATTATGTACACGCCAGAGGACACATACCTTTTATTATTACTTATAGGAAAATGAA 2940
2941 CGAGICTAAAGCTTCCACAATGTTATATGAGCAAAAACATCGAATTTTCTTAAATTAGATTTC 3000
3001 TTTAAATCAGAACATAATTAATGTAAGTTTCTTAAATTAGATTTTTAAATATGTATATATAT 3060
3061 GTAAAAATACCTTCTTTTTTTTTTTTTTTTGTGTCATCAGCATPACAGATTCTTAAATAAGTTTAC 3120
3121 TTCTTATGTTTTTAACAAATAGAAATPACTTCATCTCTTTCCTTAAATTTTAAGTCAATATCAA 3180
3181 TCCATTTCTATAATCCGGAAGATGAAGAAATCCCTTTCATCTCTAGAAAATAGGGTCAGAAA 3240
3241 GTTTTCGGTTAAAATTCGAAAGGATCATCCCTGAAGTATTTTATTGTGTTTTTTTTTATGCTTT 3300
3301 AGTCCATTAAATATTTTTTTAATGTATCGGTTTATATATATATGATTAAAGAACTTCCCATGATAA 3360
3361 ATATATTAATAAGTTTTTATTTCATTATCTATTTTATGCAACGTTTGTTCCTGCCACACAC 3420
3421 AAATGATTTAACCACACATTTTTCATAATATGGAATAAACTATAGTCTTATGTAATAATTTAT 3480
3481 GTGATATTTTAAATPAGATTATATATTTTATAGGTAATCTATTTATGCTTTTCCPATACATA 3540
3541 CAGTAGTTGTCTTAAAACATCAAAATTTTATATGTAATGTTTATTAATATGTCACACAAT 3600

FIG. 3a(6)

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3601	TCTTAAACAATATTTTTCACAACATAAAAAATAATGTTTIGACCAAACCATAATGCTCATTT	3660
3661	TTCTTTTATTTACCGGCAAAAACCATTTCTCCATTTTTTTTACITTATTAACGCTTAAGATAAAA	3720
3721	AAAAATTATTAAACAGTTTTTTTGTGTGATAAATAAAGTTTTTATATTTTCAGAAAATGTATTATAT	3780
3781	TTTCAACAACAATTAAAAATTTTGGGTTTTTTATATCTTAACATAAATGATGGCAGAGCACAGAA	3840
3841	AAATAATTGTCITTCATAAANAATTCCTCAATAAAGGAAGCTAGTGCATTTCCAAAATAAATGTTT	3900
3901	GGCTATGTGTAAGAACCAACATGGAACTCTCTGGTAGTTGTCCGGAAAAAGAAATTTTGTAG	3960
3961	TGTGCTTGTAAAGTAACGGTTCACACTAAGTGTGTTATGAATCTAATAATGTCCAACCAAAAG	4020
4021	TGTATATAAATTTTTTAACAATAAATGTCTAAATGTGTTTGTCTCAGATTTGTGGATCTATTT	4080
4081	TATAATAAATAATAATATGAATGTAAATAAATAACAAATGTGTAAAAAAGATTCGACTA	4140
4141	TTAATAAATAAATGATCACAATTATTTATAGATGTAAACCAATATTTGTGTATAAGATTCGTA	4200
4201	AAAGCTTAAGACGAGTTAAAAAGATAGAGATGAAATCACATCCCAATATCTAAATGTGAGAGA	4260
4261	TPATCAACTAACATATAAATTTTGTATATATTTGTAAAGATAAAAAATAAAAAATTTAAAAA	4320

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FIG. 3a(7)

4321	AGCAAGAGTTGATTATCAACAAGAAGTATTAGAAAGAGCAACAGTTCATCCAGAAGA	4380
4381	GTCCATGTTTGAATTTTCTTGACAGATGGGTTGCAGACAAGTCATCGGAAGGTCATACAA	4440
4441	ACTCATCAGACAACGCAAGAAAATGTATGACACAGTTTCTGAAACATGACAAAGCTCTGG	4500
4501	TCAATGAAGAACAATCTCACAAGCAGAGTTTGGGTAGACCTCCTCCAATGTCATCATCAG	4560
4561	CTACGAGCTCTGAGACTTCATCCACATCTCAGTCCCTCAGTTTCTTCCCAGGAAGTTTCCT	4620
4621	TGAGGAAGGAGGTTTATTACAGAAAATCCAAGTTACATGAGCCCTGGCAAAATCTATCAAAAGC	4680
4681	TAAGCAGAGATGATCACCAGTATTTCTTCATCGTCTTCTTCCAAAACCTGGTTTGAGAAAAA	4740
4741	ACAATCCATGTCTTTACAAACAGAGATGTAAATGTAAAGTGTGTTGTTGTTCCGATCAATC	4800
4801	TTGCAACCAGTGGAGTGAATCTATATCCACCGGTTCAAGTAAACAAGCAACTATGTGGGA	4860
4861	AAGAGGCTAAATTAACAATCGTTTTCATCAAAAGATTTGTTGCCAATGTTGTTTGGCCACT	4920
4921	TTGATTATTTPAATAATTAACCTCGGATGCTTCTGAGACAACTGTTCCTCCCATTTTITG	4980
4981	TCAATATGAACGAAGCAATGCTTTCATCTTTAGACATGAAAAGCCATTTTAAATGACCA	5040
5041	AATAACATAGTTTATPACCAAGCTTCTTATATAAATTTTACCCGTTCTAAAATGCTCTCT	5100

FIG. 3a(8)

5101 ACTATCAAAATCTAAAACTGAATTAAATTCAATTATCTTACTTGTACACAGTTTTCACCTA 5160
5161 ATCACTATTTTAAATGTATAAACTATAAAAATATAAATTAATACTTACTTAAATTTTGTAGATT 5220
5221 TAATCCATAAATTTATATTACAGTTTCAGAGATTTCATCCACATTTTCAGTCCCCAGTCCCTTT 5280
5281 ACTCATTTAAATTTTCCCTGAGGAAGGAGGTTTATACAGAAAGTCAACTTACATGAGCCTTTAC 5340
5341 TCAATCTATCAAGCTAAGTAAGAAGAGATGTCAAGGTTCCTTCTTTCATCTTTTCTGTGCCACATCA 5400
5401 CCATTTAAGTAGAAACAGTATATGTCTTACAACGGTGATGTGAATGTAAAGGGTTGTGCT 5460
5461 GGTTCGGAGTAGACTGATCTATATATCCACCAGTGCAGGAACATGGAGGCAATATGTGGGCT 5520
5521 AAAAAAACAATCATTTAACTGAATCTTTAAAGCACCTTTCAACATCTTTGTGGTTCCATGAA 5580
5581 ATACATAAATGGATTATAGGTTATTGAAGCCATTGTGTATATGTTTCTTACTTATAAAT 5640
5641 TAGTTTGAAGACAGCAATGTGTTGTTGTTGGCCACTTTTGATTTATTTAATAATTAACTTCTG 5700
5701 ATCTTTCTGAGACAATATGTTCCTCTTTATTTCCTGTCATATGAAACCAAGAGCAAAAGTT 5760
5761 TCATOCCTTAGACATGAAGGCTTATTAAATGACCACCAATACATAGTTTGTAGACGAAAGCTTT 5820

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FIG. 3a(9)

5821	CCTAATAAAATTTATTCTCACTATCTAAAATCTAAAACCTGAATTCAAATTCATCTATCTGAT	5880
5881	TATTATATAGTTTTCATTTTTTIGTTAATTTTATIGAATGAGTAAAAAATTTAATTAAATAC	5940
5941	TTACTATTTTTTTCATATAATCTTATAAATTATGTTACGGTGGGACATCCACCTAATAA	6000
6001	CCTATTPAATTTTAAATAGTAATATTTTIGAAAAATATTTTATTTGACATTTGTTTAAATAAAT	6060
6061	CATAATCTTAAAAATAGCAATAACTTTTTTIGAAAAACATCAGATTTTATGTACACGCATAGGA	6120
6121	CACATACCTTTTTTATTACTTAAAGGAAAAATGAACGAGTCTAAAGCTTCCACATGTTATC	6180
6181	TGAGCAAAACATGGATTTTTCCTAAATTAAGATTCGTTTAAATCAGAACAATTAATGTGAG	6240
6241	TTTCTTAAATTAAGATTTTTPAATAATGTAATATATACGTAAGAATACTTCTTATGTTTAAAAA	6300
6301	AAAAAATAGAAATACITTTATCTCTTTCCTAAATTTTTPAAGCCCAATATCAATCCATTTCTAT	6360
6361	AATCCAAGATGAAGAAATCCCTTCAACTCTCGTTTACGTTCTTAAATTAATCTCCATCTTC	6420
	M K K S L Q L S F T F L I I S I I L	
6421	TCCTACAAGGTTTGTATTTACATCTTTPAATATATTTGTCATATATAGTAAATTCATATAAATTTG	6480
	S Q	
6481	ATTATACTAAACTTTTGACITTTTAAATAATTTGTAAACCCCCCATATATATAAATTTTATTTTA	6540

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6541 CTATATAAAACATAGCATTAATTAATCTCTTTGTGTAAATTCATAACTTTGCAGAAGGG 6600
6601 AAGAAATATAGAAAGTATGGTCAGAAATGTTTGGGTTAATATTGAAGAATCAACCCCTG 6660
6661 AAGTATTTAACTGTTTATTATACCTTTATGCCATTTTATAATTTTTTAAATGTATGGTTTA 6720
6721 TATATGATGAAGAGCTATTATGATPAAATAATATTAATAATAGTTTCATTTTTTATCATCTPAT 6780
6781 TTACGAACATTTTGTCTTCCACATACAAAATGATTTTAACCGACATTTTTTCATAATATGGAT 6840
6841 GAACTATAGTTCTTACGTAAATTTTATTGTGATATTTTAACTAATTTTTATATATTTTTTATG 6900
6901 CTTTTCCAATACATACAGTAGTTGTTCTTAAATATCAAAAATTTTATACGTAATGTTTAT 6960
6961 TAATATGCACACAATTCITAAACCATATTTTTTCACAAAATAAATAAAGTGTGACCA 7020
7021 AACGATATGCTCATTTTTTTTATATATACIGGCAAAATATATTTTCTTTTTTTTTTACITTA 7080
7081 TAACGTTTAAAATGAAATGTTTATTAACAATTTTTTGCIGATAAATAAATTTTCTATTICA 7140
7141 TAAAATCTATATATATTTTCTAACAATTAAATTTTGAATTTTAAATATCTTACAGGAATG 7200
M
7201 ATGGCAGATGGCAGAAAAGAATTGTCCTCGTAAATTTCCAATAAAGGAAGCTATTGT 7260

FIG. 3a(10)

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M A D A Q K K N C P R K I P I K G S Y C

7261 GCICCAACTATATGTTGGATAAGTGAAGAAGCAACATCGAACTGTGGTAGTTGTCCG 7320

A P T I C L D K C K K Q H G T V G S C A

FIG. 3a(II)

7321 GAAGAAAAGGATTTTGTAACTCGCTTGTAAAGTAAGAGTTCCTCACTAAGTGAATGAAT 7380

E E K G F C N C A C K STOP

7381 CTAGTAATGTCACCAAGTTTATATTATTCTTTTAAACAATAAGTCTAAATGTTTGT 7440

7441 CTCAGATTGTGGATCTATTATAATAATAATTATATGAATGTTAAATAATACAATTG 7500

7501 TATAAAACAAGAGTGGACTATTATAATAATAATATATGATCACAGTATTGTTAGAAGTAACCA 7560

7561 ATATTACGTGTAAATCAAAAGCTTAAAGACTAGTTAAATAATATAGAGATGAATCACAAAC 7620

7621 CAATATTTTAAATGTTATATAATCAACTAACATGTAATTTTGTACACATTGTAAAAAATAA 7680

7681 AAAAAAAAAAAGCAAGAGTTGATTAAACAAACAAGAAAGTTTAGAAGAAGCAACAGA 7740

7741 TCATGCAAGAGCAGTCTAGGTTTGAATTGGCTTIGACAGATGTGTGTCAGACATGCCCATGA 7800

7801 GGAAGTCTTACAAACTCATCAGACAACAACAGAAAAATTGATAGCATTGTTTCTGAACAT 7860

7861 GACAAAGCTCTGGTCATGAAGAAAAATTTCACAGCCCAAGTTTTTGTAGACCTTCTCCCA 7920

FIG. 3a(12)

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FIG. 3b(1)

-789 TTTTTCACAAACACATATGGAGCCAGCANNCTATCACCCICCGCCACGTGCACAAGGATCAACICCTTAAGCTC
 -709 CTTATCTPACGGACTCATCTTAGTTATCTTAACCAAAATATTAATTATTAATAATTCATATCCATCGGTTTACCTGCTAAG
 -629 GACTCCCAACGTCGCTCCGTTGCATATGCTCTTATPAAACTTAACTTAAGTGGCGCCPAAACGGTCGTGTATACCATTTTT
 -549 CTTGGAGTTAAGTCACTATCTTTCTTCATTTTCTTTTCATCTTAUGTTTCTTTTGTGGCCGCGAATACAAATGATGTGGCGG
 -469 ACTCCCTTCGCGAAGTCTGCTCTCCAGAACTATGTAATAACAATTTTCTTTTAGATTTTATTTATTCAGTTGGTTGACAAAA
 -389 AAAAAAGAGTGTTTAAACCAAAAAAACAATAATAATAATAATAATAGTTCTGTAAATTTATCAGTTTCCATG
 -309 TGAACCTTCTTGAAATACATTTCAAGTTTATGTTCTATPAACTTATTCACGTGACTAATAGATTTTCTCTCTCAGAGTATTTCT
 -229 TTTAAANCCAAATATAGCCCAATTTCTCAAGACATGGAAACCCAAAGCCAAAATTAGAAAAGGGAGAAAAATGTTAAAGAG
 -149 AACTAAAAACACTAATAATAGCGAGAGGTTAAACTTCAATGTCTCCAAATATGGAATGAAATAGCGAATGAGAGGAGA
 -69 TGCCAAAGAAAATCAGAAAACGAGTCTTAAAAACCCCTATATAAAACGTCTCAAGCATTTTTCACAACTTCGAANTTCAAA
 12 CCCCAGAACATAACCAATAAAAAATAAACATTAATATACCCCAAAAAACACATATGCGAGGGGTTAAACTATTTGGCCGCG
 M R G V K L L A A
 92 TGCCCTACCTGGCCGCGAGCCGCAACGGTGGTGGTCCATGCGCGAGACCCCTTACTTCCACCACGTTTGGAAACGTGACCTA
 C L Y L A A A A T V V V H A E D P Y F H H V W N V T Y
 172 TGGACCGCTTCTCTCTAGGGGTTCCACAAAGTCAATCTTAATCAACGGCCCAATCCCTGGTCCCTAACATCAACTCAA
 G T A S P L G V P Q Q V I L I N G Q F P G P N I N S T
 252 CCTCCACACAATGTCAATCATCAAGGCTTCAACAAACCTTGAATGAACCCCTTCTCTCTCACTTGGTAAATATTAATAACCA
 S N N N V I I N V E N N L D E P F L L T W
 332 TTCTTCTTCAAAACATATCTTTTCTCTAGAAAAAAAAGAACTCTTTAGCCCATGGTCCGAATCTAAAATTTAGAAAA
 412 CATPAACTAAGGAGTCTGATCATTTACATTTATTAATAAGTTATAATATATATTTTTCATTTTAGGAATGGAATCCAGCAC
 N G I Q H

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FIG. 3b(2)

492 AGGAAGACTGTTGGCAAGATGGAATCCCGGGACTATGTGTCGATCATGCCCGGCAACACTACACTTACCATTTCCA
 R K N C W Q D G T P G T M C P I M P G T N Y T Y H F Q

 572 GCCTAAGATCAGATAGGAAGCTACTTCTACTATCCACCACAGGGATGCACCGTCCGCTGTGGATATGGTGGACTCC
 P K D Q I G S Y F Y Y P T T G M H R A A G G Y G G L R

 652 GAGTGAACAGCCGTCTCTCATCCCGGTCCCTTACGCTGATCCCGAAGATGACTACACTGTCCTCATCGGTGACTGGTAC
 V N S R L L I P V P Y A D P E D D Y T V L I G D W Y

 732 ACTAAGAGCCACACCCAGTTGAAGAAGTTCCTCGACGGTGGTGTACTATTGGTCTCCAGACGGTATTGTCTCATCAACGG
 T K S H T Q L K K F L D G G R T I G R P D G I V I N G

 812 AAAGTCCGAAAGGTGATGGAATCAGACGACCGCTCTTCACTTCAAGCCCTGGAAGACTTACAGGGTTAGGATCTGTA
 K S G K G D G S D A P L F T L K P G K T Y R V R I C N

 892 ACGTGGGTGTCACACATCTATCAACTTTAGGATTCAGAATCACAAGATGAAGCTGTTGAATGGAAGGATCGCACGTT
 V G V K T S I N F R I Q N H K M K L V E M E G S H V

 972 CTTCAAACGATTACGACICTCTTTCAGGTTACGTTGGCCAGTGGCCAGTCTTGGCACCATCGTTCACCGCAATCAAGAACCTAA
 L Q N D Y D S L D V E V G Q C F G T I V T A N Q E P K

 1052 AGATTACTACATGGTTGCATCCCTCTAGGTTCTTGAAGACGGTTATTCACAACCGGACTTCTCCGTACGAGGGAGGCA
 D Y Y M V A S S R F L K T V I T T T G L L R Y E G G K

 1132 AAGGACCCGCCCTCTACAGCTTCGGCTGGTCCGGTGGATGGCCCTGGTCTGTTGAACAGTTCCTCGATCTCAGGTGG
 G P A S S Q L P A G P V G W A W S L N Q F R S F R W

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FIG. 3b(3)

1212 AACTGACCGCTAGTGCAGCTAGGCGCTAACCTCAGGGATCTTACCAATTATGGAAGATCAACATCACACGCAATCAA
 N L T A S A A R P N P Q G S Y H Y G K I N I T R T I K

1292 GCTCGTGAACACTCAAGCGAAGGTGCATGGTAAGCTTAGGTTTGCAATTGAACGGAGTCTCCACACAGAACCTGAGACCC
 L V N T Q G K V H G K L R F A L N G V S H T E P E T P

1372 CTCTGAAGCTGGCCGAATPACTTTGGTATTTCCGACAAGGTTGTTAAGTATGATACCATCACCGATGACCCCTACCCCGGAA
 L K L A E Y F G I S D K V F K Y D T I T D D P T P E

1452 CAGATCAAAACATCAAGATCGAGCCTAAGTTCTTACATCACCCTACCTTCGTCGAGGTTGTTTTCAGAACCA
 Q I K N I K I E P N V L N I T H R T F V E V V F E N H

1532 CGAGAAGAGTGTTCAGTCTTGGCACTTGGATGGTTATTTCTTCTCTCGTTCCGTPAAGTAAACAACACACACTTTGT
 E K S V Q S W H L D G Y S F E S V A

1612 TTCTTGCATCACAAGTAACCTCTTCATNGTAACTTAAGTTTACTTACTTAAAGTTGAGCCAGGACTTGG
 V E P G T W

1692 ACCCCAGAGAGAGGAAGAACTACACCTCTCTTGGATCGAGTCAGACAGACACAGTTCAAGTCTACCCAAAGTCTGGGC
 T P E K R K N Y N L L D A V S R H T V Q V Y P K C W A

1772 AGCAATCTTGGCTACATTTIGATACTGIGGAATGGAACGTTCTGTTCTGAGAACACAGAGACAGCTTACTTAGACAGC
 A I L L T F D N C G M W N V R S E N T E R R Y L G Q Q

1852 AGTTTACGCCAGTGTCTTGTCTCCAGAGAATCATTAGAGATGAATACATGCTCCCTGAGACAGCCCTCCATGTGGTC
 F T P V S C L Q R N H L E M N T T C L R Q A S N V V

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FIG. 3b(4)

1932 TCGTCAAAACACACCTAAACCTGTAAACCTTACGCTGGTGCCTAAGTTTAACTTTTAAATACAACATAAGAGTTTNTGA
S S K T H L N L L T L T L V P K F N F *

2012 NICTTCTGTGATNIGAAAATTAAATTNCTAAAAATTATATGGTTTACTCTGTPATATACATGGGAANTIGAATAATGTATGTATGT
2092 GTCTATACCTTTTAAAGTAATTTTTCITTTCTTTCACAAGAAGCATTTAGTCTTCTCTTTTGGTTTMMGTCTTTCACCTTTTGTGA
2172 ACAATTATGTAAUTGTAATTATGTATCCGATAATCTTCGATGAATAAGCAAAGAGATCTTATTTCTCCCAAAAATAAACT
2252 TTACAATAAAAGTATTTCTCTATAGCCTAGAACCATGTATGATGATAACAAACCCCTTCTTCTTAGTCTGAGCCCTTTTTC
2332 TAAGGCTCTTTTATCTCGTGGTTCTTCCACCAACCGNGITTCCTTGAGAGAGAAGTTCATCGAGGCTATCATCTCTCTGT
2412 ATCGACCTGAACAACCTCTTGAACATCGCCATTACTTTGAATCATAGTTGGTCTTTTGAACGGTCTGATCGTCCAGACACT
2492 GAGACCGCAATCTTCAAGTAATGAACAACAGCTCAACGTCCAGATTTCTCAGTTATCAGGTCCGAATCAAGAACTCAGCT
2572 CCTCTACTCTCTTATAAAGCTGTTTAGCCCATCCACGAGATTTGTGTCTCCACCAAACTCTCTGATCGATCGGTTT
2652 CTTCCCGAGAGAGAGCTCCAGAGTATAACCCCGTAGCTATACACATCTCTTTGGTCTGCCACCTGAAACCTCTGGTAAT
2732 ACTCTGGCGGAACGTAAACCGGAGTTCCCGGAGGTGTACCGCTCAAAATGCGGTGTCCAGAGCGCTCACCAGCCCTTGCCA
2812 TACCCAACTCCGAGACCGGTGCTGTGAAGTCTTGGTCTAGA 2852

FIG. 3c(1)

1 GAATTCCTCAACANNIGATTCTCATCAACGACAGTCCCTGGTCCCTAACCTAAACCTCCACATCCACAAACAATGTCGTC
 I P Q X X I L I N G Q F P G P N L N S T S N N V V

81 ATCAATGTTTCAACAACCTTIGACGAGCCCTTCCCTCTTIGACCTGGTTAGTCACCATTTCCCTCTCATTTTATAGGCATTCT
 I N V F N N L D E P F L L T W

161 GTTCTAAAATTTAAATGATATTTTAAAGCTACAAATTTTTTTCATTTTTTAAATATAACIGTCTTTTACAAAGGCCAAA
 241 CGAGAAATGCAAAATGGAAGTCTCATTTTCGTTTIGATPATTTTCATTTACAGTTAACATPATGATTTTTTTTTTTCAGATCTT
 321 TGTAGGTTTGTGAAAAAAGTTTGTGATAGTTATGTTATTTTGTCTCCCTGGATCTTPAGGTTTGAATAATTTCATAACCA
 401 AATTGAAAAACAATAACTTTTGGATCGTTAATCAAAATCTCTCTTTATTTTTTAAATGTTATATTAATTTTCACATAAATTAC
 481 ATCTATATATATATATAAATACAAAATAAATGATAATTTTCAGTTTATCATATATTAATCAGACTPAAATAATAATAAAC
 561 AAAAAGAAATTTAAACACATTTTGCATCAGTTTTPAGATTAAAGATTGGTTATATATTACCAAGTAATTTATGCTAGTCTTCA
 641 TTGCAAAATTTAAACACATTTTAACTATGTCGGTTGCGGTGACATGCGAGGATGGTCTCCAGCACAGGAAGAATCAATGCGC
 S G L Q H R K N S W Q

721 AAGATGGTGTGACCGAACCTCATGCCCCATCCAGCAGCACCACTPACACTTACCANTTCCAGCCTAAGGACCAGATC
 D G V T G T S C P I P A G T N Y T Y H F Q P K D Q I

801 GGTAGCTACTTCTACTACCATCAACCGCCCTGACCGTTTCTCCGGTGGTTTCGGTGGCCCTCCGTTCAACAGCCGTCI
 G S Y F Y Y P S T A L H R F S G G F G G L R V N S R L

881 CCTCATCCCCNCCCTTACGCTGACCCCGAAGATGACCAACCATCTCTCATCAACGACTGGTACCAAGAGCCACACCG
 L I P X P Y A D P E D D H T I L I N D W Y T K S H T A

961 CTCICAAGACCTTTCCTTGACAGCGCGCCGCACTCTTGGTTCCCTGACGGTGTCCCTCATCAACGGAAGTCCGGTAAAGTC
 L K T F L D S G R T L G S P D G V L I N G K S G K V

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FIG. 3c(2)

1041 GGAGGACAAACAAGCCCTCTCTTACCATGAAGCCAGGAAGACCTACAAGTACAGAATCTGTACGTTGGGTCAATC
 G G Q N K P L F T M K P G K T Y K Y R I C N V G F K S

 1121 CACTCTTAAGGATCCAAAGACACAAAGATGAAGCTTGTGAGATGGAAGATCTCAGTCTCTCCAGAACGACTACG
 T L N F R I Q G H K M K L V E M E G S H V L Q N D Y D

 1201 ACTCGICGACGTCCAGTCCGACAGTCGTCGTCGTCTGTGACCGCTGACCAAGAGCCAAAGAGCTACTACATCGTT
 S L D V H V G Q S F A V L V T A D Q E A K S Y Y M V

 1281 GCATCCACTAGGTTCCTCAAGAAGGAAGTGACCACTGTGGTGTGATGAGCTACGAAGGAAGCAATGTTCAGCCTTCAAA
 A S T R F L K K E V S T V G V M S Y E G S N V Q P S N

 1361 TGTCTTCCCAAGGCTCCAGTTGGATGGGTGGTCTCTTAACCAGTTCAGATGGAATTAACCGCCAGCG
 V L P K A P V G W A W S L N Q F R S F R W N L T A S A

 1441 CCGGTAGGCCTAACCCGCAAGGATCTTACCAATTACGGAAGATCAACATCACACGTACCATCAAGCTCGCCACACCAAG
 A R P N P Q G S Y H Y G K I N I T R T I K L A N T K

 1521 AACTTGGTGGACGTAAGTTCAGGTTTGGCTTAACGGTGTATCACACGTTCACACACNAGACTCCCTTGAAGCTTGTCTGA
 N L V D G K V R F G L N G V S H V D T X T P L K L A E

 1601 GTATTTCNAGATGTCGAGAAGGCTTCAATACAAATGTCTATCAAGGACGAACAGCAGCCAGATCACTACTAACC
 Y F X M S E K V F K Y N V I K D E P A A K I T T L T V

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FIG. 3c(3)

1681 TTGAGCCTAATGTCCTTAACATCACATTTCGGTACCTTTGTGAAATCGTCTTCGAGAACCCAGAGACGAGCATGCAATCA
E P N V L N I T F R T F V E I V F E N H E K S M Q S

1761 TTCCATTTCGATGGTTACTCCTTCTCTCAGTCGCGTAAAGCTTCATTAATAACTCTATAGGCCAATGTTTCACCTTANTAN
F H L D G Y S F F S V A

1841 GCGCAGAACCGCGGTGATCTTTTACTTCAGATATAAGATTCCTAACAATTTTTTTAATATTTTTTCAACAGTTCGAGCC
S E P

1921 AGGAACATGGACACCCAGAGAAAGAAACAACTACAACCTTCGTCGATCGCGTCAGCAGACACACCGTCCAAAGTNTTCCCA
G T W T P E K R N N Y N L L D A V S R H T V Q V F P K

2001 AGTCGTGTCGCCCATCCCTTCACATTCGACAACGCCGGTATGTGGAACATCAGATCAGAGAACTGGGAGAGAGATAC
S W S A I L L T F D N A G M W N I R S E N W E R R Y

2081 TTGGGACAGCAATGTACGTCAGTGTCTTTCCTCCCTGAGAAATCACTAAGAGACGAAATACAACTCCACTCAACACCAA
L G Q Q M Y V S V L S P E K S L R D E Y N I P L N T N

2161 CCTTTGTGATCGTTAAGGCTTGGCATTTACCTACACCCCTACACATTAATTAATAACTCATTCCACAAAAGTTTTATT
L C G I V K G L P L P T P Y T I *

2241 TATTATTGATATATGFAAATTCCTACTTTTTACAAGTCAGTGTATTACGTGACTAATTAAACCTTTCCTTAATTCATT

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FIG. 3c(4)

2321 TAACATACTACTATAAATTACAGATCCCATTTGTTGTTTCACTAATAGTAATATATACAAACATTTAGCTTACTTAATAT
2401 AATCCTCGATTTCTAACACAAAGAGCTGTTATTCATTTCATATAATGAACAAAACCTTGTTACCCCCCTATGGTGAACCCCTCTAA
2481 TTCACCTCTATTCTTAACACCAATCAAAATIGACATGTAAGATTAAATAAAAGAAATAAATTTAAAGAAAAAATAAG
2561 GTTTCCTAAAAAAGGGTTTATTGTTCAATAACCAAAAAAATAATGAAAAATTANATTTTGGGAGAGAGAGTAGAGAGAGAT
2641 AGGAANAAAAAGTAGGAGAGAGAGAGAAATTTAGTTAGTTAGTTAGTTATTTAAGTTTTTTCATGTATATAGGGTGCAATT
2721 TCCCAAAAAAAGTCTCGGTTAACAAAGGAAATAACGTGTTGTTGTTACCGCGTCCATCGACGTCCTTCTTCTTTTATA
2801 GCCCAGAGAGAGATAGAAAGCTGAGTCTCCAGTCTATGCTTCCAAATCAAAATTTTCGATTTTCATCTTCTCCAAATCAAAATC
2881 AAGCCATGTTGCCCTTATGAGTAGAAGAGCGCTGATGGTGGAGATGAAGATGATGGTATGGGATTCAAACTTGCAGCGCCC
2961 CAGGGAGGAAGAGATGACTTTACTTGTGAACCAAGNCCTGTCCTTAAATTTCAACAAAGAAACCGATGCGCAATTGGAAAT
3041 CAGACACTACAACCTTGNITTAATAAANAACCGCTANTTGTAAATGTAATCTTGAATAATAGACTTTCGCAAGTTGATGAGAACTC
3121 ACTGATGCAAGTGTGCGTATCTTTGATGTAGTAAGGTCAACAGCACTTTTAAATGTATTTCTTAAATAATAGATTTTGC
3201 AAGTTCTTTGTTGTATTTTCTTCTTCTTCTGTTGGGACATCTGACCTGTAAATCAGAGATATATATCCCAACCAACCAAGGTT
3281 TCCGTGTTTAATT 3293

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FIG. 3d(1)

-2022 GGATCCGTTCCCTTTCCGGTCAATTTTCTCTCTCCGTCATAGGAGAAACATCCGGATCATGATCACTCCGATCAAACTAGCCT
 -1942 TTCTCTTATTATTATTCATCCACAAACAGCAACCGCTGCACCCAAAGCCAAACGGCAGGAACCTACCTTTTTCACACCGCATGCA
 -1862 AAGCAGTCGCGGAATATGCACTGTGTCTTAACAACAAGTCCATGCTGTAAACCCCTTAAACACGTTCCCTACCGAT
 -1782 GACCCCTATCGAATTGATCCGAGCGTTAGCGCTTCGGACTGAATCTTCTGTGAAAGAAAGTGTGGTTTCTCTCTCCGAGAT
 -1702 CAACCAAAACACAAATCAAAACCGCAACCGCAGCTCCAGTGGTCAACAGCTCCGAAAACAACTTGAAGTACGCATTAGAAG
 -1622 ATTTCACCTGATTTTGGCAACGCTATGCGGAAAGATGTAAAGACGTTGGCTCATAACTATTTCACGGTGTAAAGAAAGTTA
 -1542 ATGTCGATCATGGGTACCATTTGGACCTTCTTTGGATGATATTGATGATPAGAAATCTGTGAAGGAANTGGAGACTGGGAT
 -1462 TAGTGTTCGGAAGAATCTAAGCAGTAATACGTAATGATGTGTTTAAATGCTTTGAAAACATAATTTTAAAGACGTTTGGTATCA
 -1382 AGGTCAAACTTAAACGAGGAAGACACATTCGCCCCAACCGCCACCATTGTTCGAATTTATTTACTACTGATTAATGATTTGGAT
 -1302 GTAATAAATAATTAATATACACTCGTATAATGCCCTAAAGCCACCATAATATGPACGATGTATGTTCTAGGTTTATATACATGT
 -1222 ACCGATGGATTTCCGAGATGATTGCAAGGATATATTAATGGAATGTGAGTTTATTAATAAGTTTGTGAATTTGTCATCAAA
 -1142 ATGAAGCTGAAAGTATATATGTTGATTACATAATTAATTTGATAGACCTTGTATAACTCGAACAATAATATACTATCGCAATCA
 -1062 TTATTGGTTAAATGTTTCCGATAACATGTCCTACGTCAAACAAATAATTTGTGCAATTTTCAATATATTGATGTCATGAAA
 -982 GTTTCATATATTGATGATGAACCATGCAATTTGTTTCCCTTTGTCCTCCCGGACTTTGTCAACATTTCAACGAACCTTTCAGT
 -902 AATCAACTACATATGATCTGATCTAGATGATGTATGTACATGTACGACCATTCCATGAATCAACCCGATGTTTCTTTTAT
 -822 CAAGCGGATGTTTAAATGTACCCCGCTTTCTCGTTCTGTTAGGTATAAATACGAGTGAATAACATTCCTATGTATAGT
 -742 ATAGTACTTCAATTTTGTGTCACCAAAATTTAAATATATAGGACATAATTAATTTTCTCAAAATGAATTTTGAGTTTTCAGTG
 -662 CCTCCTGAAAGATGATTCACAGCTTTCAATTTTACAATGCTGGTCAACCCCCCAAGTATTTATCGTTATATAAAAATAC
 -582 TCAGAAATAATCTCACACCTAAACACTCGCGCATGAACCTTGTTPAATAGTCTAAAAAGATGTCCCATGPACTGAATGAATATAC
 -502 CATTCATGTTTIGAGTAAACACCATAGATGACACAAATTTTCTTACTTACCACATAATAGATGACACAATTTGTTCTTTTGTTCATG
 -422 TTGTPAGTGTCCGATTAATTTGGGTTGAAGAAGATGAGAACTCTCGATGAGAAAGCTTTAGAAAACATGCAATTTTACTTTTCGTT
 -342 ACTTTCCGAAATTCGGAAGTAAACTAGTACTAGGAATGAATAATCCCAAATAAAGTTGTTTATTTCTCTCTAATCCTAAAAT
 -262 TAATAAAATTAATAATAGACTAACTTTTCCATCCTTAAGTTACTTTCTTATTTTTPAGTAAATCGAAGCTACACCTCTTGATCA
 -182 GGACAAAGACATAATACCAATCACTCTTGTGTTGGTGAATAATTTTAAATCTCAAAATCCCAATATTGATTTAGAGAAAGTTTCAGCC

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FIG. 3d(2)

-102 ATTCAACTACCTPAAATGTCCTCCCTCCATGCAAGCTCATGCGGAACCTAAATTTT¹PAGAACTPACA²CTTCTATA³AGAATCT
 -22 CCCATAATGCACCCATAATCTCCACCAGCCCCCATTAATAATPATCCACCGGTCTGTAATAATAAATAAGTCCCCCTCCCT
 59 CTCCATTTACCTCCTAAATAAAACCTGAGGAGAAAACAAAAAACAATAAATAAGATTAAAAAATAAATAATAG
 M
 139 GCGGTCGGGAAAATTGTGATATACAGTGGCAATCCATGCTTCTPAGTGGTGGTGTGGCCATAGGAGTGTGTCACCTTTGTAA
 A V G K I V I S V A S M L L V V G V A I G V V T F V N
 219 TAAAGGTGGTGGTGCAGGTGGCGACAGACCTCTGA⁴CTCCATCAGAAAGCGGTGAGTCACTTTGTGGTCA⁵GCCACAG
 K G G A G G D K T L N S H Q K A V E S L C A S A T D
 299 ACAAGGTTCA⁶TGCGCAAAAACNTTGACCCAGTCAAAAGCGAGCATCCAGTA⁷AACTTATCA⁸AAAGCCTTCATGTAGCT
 K G S C A K T L D P V K S D D P S K L I K A F M L A
 379 ACAAAAGATGCTGTCA⁹AAATCCACA¹⁰AACTTCACGGCTTCAACCGAAGPAGGTATGGGAAAAACA¹¹TAA¹²CGGACGAG
 T K D A V T K S T N F T A S T E E G M G K N I N A T S
 459 CAAAGCGTTC¹³TGATTACTGCAAGAGAGTGTCTGATGTACGCTCTCTCGAGGATCTCTGAGACCATGTGTGAAGA¹⁴ATGGGTG
 K A V L D Y C K R V L M Y A L E D L E T I V E E M G E
 539 AAGATCTTCAGCAGATGGGAGTAAGATGGACCAGCTTAAACAATGGTTAA¹⁵CGGAGTTT¹⁶TAA¹⁷TACCA¹⁸AACCGATTGT
 D L Q Q S G S K M D Q L K Q W L T G V F N Y Q T D C
 619 ATTGATGATATTGAAGAATCGGA¹⁹ACTAAGAAAGTCA²⁰TGGCGGAGGAATCGCTCACTCCAAGATT²¹TGTCCAGTA²²ACCG
 I D D I E E S E L R K V M G E G I A H S K I L S S N A

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FIG. 3d(3)

699 TATGATATCTTCCATGCTCTAACCACCGCAATGTCCCAAATGAATGTTAAGGTGATGACATGAAGAAAGGGAACCTCG
 I D I F H A L T T A M S Q M N V K V D D M K K G N L G

 779 GAGAACTCCAGCTCCTGATCGTGTATCTTCTTGAAGACTTGGACCAAAAAGGATTACCTAAATGGCATTTCTGACAAAGAC
 E T P A P D R D L L E D L D Q K G L P K W H S D K D

 859 AGGAAGCTTATGGCTCAGGCCCGACGCCCTGGTGCACCTGCTGATGAAGTATCGGTGAAGCGGGGGTGGTGGCGGTAA
 R K L M A Q A G R P G A P A D E G I G E G G G G K

 939 GATCAAGCCGACTCATGTGGCTAAGGACGGAAGTCGACAGTTTAAGACGATTTTCGAGGCGGTTAAAGCTTGTCCGG
 I K P T H V V A K D G S G Q F K T I S E A V K A C P E

 1019 AGAAATACTTCGACGTTGCATTATCTATATTAAAGCTGGTGTCTACAGGAACAAGTCACTATCCCTAAGAAGGTAAAC
 K N P G R C I I Y I K A G V Y K E Q V T I P K K V N

 1099 AAGCTTTTCATGTTTGGTATGCTGCAACACAGACAATCATTAATTTTTCGAGAGTGTGTTAGCCCTCGAACCAC
 N V F M F G D G A T Q T I I T F D R S V G L S P G T T

 1179 TACTTCACICAGTGGCACCGTTTCGTAAGTCTCATTTAATTAATCTTGTCTTTTAAATTTTCCATCTAACTAAATTGCAC
 T S L S G T V Q

 1259 CGTGCAATATCTAATATPAGTTGGTATCTPAATATACATGCAAGTTGATATCTAATCATATACATGCAATGCAG
 1339 AGGTGATCTGAGGGATTCATGCGGAAATGGATCGGGTTTCAGAACACTGCTGTGTCATTAGGACACCAAGCTGTCCGG
 V E S E G F M A K W I G F Q N T A G P L G H Q A V A

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FIG. 3d(4)

1419 TTCCGTGTGAACGGAGACCGTGGGTCATATTCAACTGCAGATTTCACGGTTACCAAGACACGCTCTACGTCAACAACGG
FRVNGDRAVIFNCRFDGYQDTLYVNNNG

1499 ACGTCAGTCTACAGGACATGTGTGTATCCGGTACAGTCGATTTCATCTTCGGAAATCTCGACCGTGATTCAAACT
RQFYRNIVVSGTVDFFIFGKSA TVIQNS

1579 CTCTATCCCTCTGCCGAAGGGAAGCCCCGGACAAACCAACCACGTCACAGCCGACGGTAACGAGAAGGTAAGCGGTG
LILCRKGSPGQTNHVTADGN E K K A V

1659 AAGATTGGTATCGTTCTCCATAACTGCCGTATCATGCGCGACAAAGAGCTCGAAGCTACAGGCTAACCGTCAATCATATA
KIGIVLHNCRIMADK E L E A D R L T V K S Y

1739 CCTTGGACGGCGGTGGAACCATTTGCCACCGCAGTTATCGGAACCTGAGATTGGCGATTTCATCAACCGACAGGAT
LGRPWKPFATTA V I G T E I G D L I Q P T G W

1819 GGATCGATCGCAAGGAGAAAATTCATTTCACAGCTACATATGTTGAGTTCAATAACCGTGGACCGAGGCTAACACT
N E W Q G E K F H L T A T Y V E F N N R G P G A N T

1899 GCTGCGAGGGTTCCTTGGGCTAAGATGGCTAAGTCTGCTGCTGAGGTTGAACGTTTCACCGTCGCTACTGGTTGACTCC
A A R V P W A K M A K S A A E V E R F T V A N W L T P

1979 TGCTAACTGGATTCAAGAGCCACGTTCTGTCCAGCTTGGATTATAGAAAACTAACPACAAATATATAACGAATA
A N W I Q E A N V P V Q L G L *

2059 ATATATAGTATGATCATGTAAAGGTAAAGTACCATACGACCTCGCTCTCCGGGATCAGGGCTCTTTTTCGTTATTATTAG

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FIG. 3d(5)

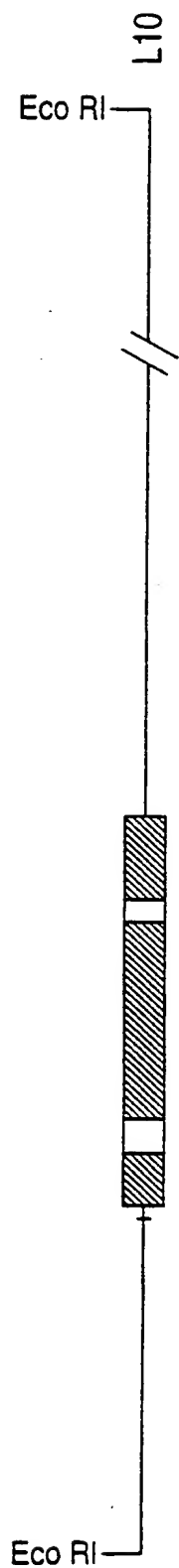
2139 GGTCTAGCGGTTTIGGGATGATGTTTIGTATAAGATTGCTTTTIGTTTTCACATGCGCAAAACATATATACAAATATCTTTATT
2219 TCCTCTTTTACTTTCTTTTATTCAAAATAATGAGTTTTPATAACCATGTTGATCTCTATATATTATAGACATCCCTATCCCTA
2299 AACATGATAAATATAACTTTCAAACTAATACCCCTAGTAATAATATATATAACAAATAATACCTTATGTAAGATTATGTGCAA
2379 GCATGGCTCAACAAATATAACTTTTATATAAAGTTTGCATTATTTGCTATGTTTATATATATGATTGCTATATGTAAGTAAGTT
2459 GATAGAAGTTTATGAGGACAGAGATGACTATTGGCCAAATATTAAATGAGAAGTGTGATATGTTATTCATTATCAAAACAAA
2539 GCAAGCCCTATATTACCAAAATCAAAATCTCACTTCAAGCGAAGCTGCCCATTTGATGATCTCACCAATATTTTACACACATTA
2619 CAACGTGACATTATCTCTCTCTATGCGCTGTTTCTTAAACCAAGTAACACAGAGTCCAAATCCAAOCTTCCAAACCCAA
2699 CCCCATTTCATCTTATTTGGGATCTGTCTTGAATCGATAACATTTCCGGTTTAAAGATGTTTTCGCAAGGACAATACATCTCTT
2779 TACGCAATGGACCTGAATGCTGCTAGTAGAGTCAATAAAGATGCAATCGGCTAAAGCCAGTTGTCTCTTACTATCAATTGTATA
2859 AGATCCTAAGGTCCCTGCTATTGGAATGAAGCAACAAGTTCCCTAGACGCCCTAATCCGACTATGTGGTCCCAAGATTCACTGG
2939 ACCGGTTATGCTTGAC 2955

FIG. 4

Bp4C	AAAAAATAAGAACTACTTTATCTCTTTCCCTAAATTTTAAAGCCAATATCAATCCATTTCATATAATCCAAAGATGAGAAATCCCTTCAACTCTCGTTTA	100
Bp4A	T	T
cBp401		
cBp405		
Bp4C	CGTTCTTAATTATCTCTCACAAGGAATGATGGCAGATGCGCAGAAAAAGAAATTGTCTCCGTAAAAATTCCAATAAAAGGAAGCTATTGTGC	200
Bp4A	T	T
cBp401		
cBp405		
cBp408		
Bp4C	TCCAACATATATGTTGGATAAGTGTAAAGAACACATGGAACTGTGGTAGTTGTGCGGAAGAAAAAGGATTTTGTAACTGCGCTTGTAAAGTAAGAGTTTC	300
Bp4A	T	G
cBp401		
cBp405		
cBp408		
Bp4C	TCACTTAAGTGTANTGNAATCTAGTAATGTCCAAACCAAGTTTATATATTCTTTTAAACAATAAGTCTAAATGTTTGTCTCAGATTTGTGGATCTATTTA	400
Bp4A	T	T
cBp401		
cBp405		
cBp408		
Bp4C	TAATAAATAATAATATGAATGTTAAAT	427
Bp4A	T	
cBp401		
cBp405		
cBp408		

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FIG. 5



DNA SEQUENCE

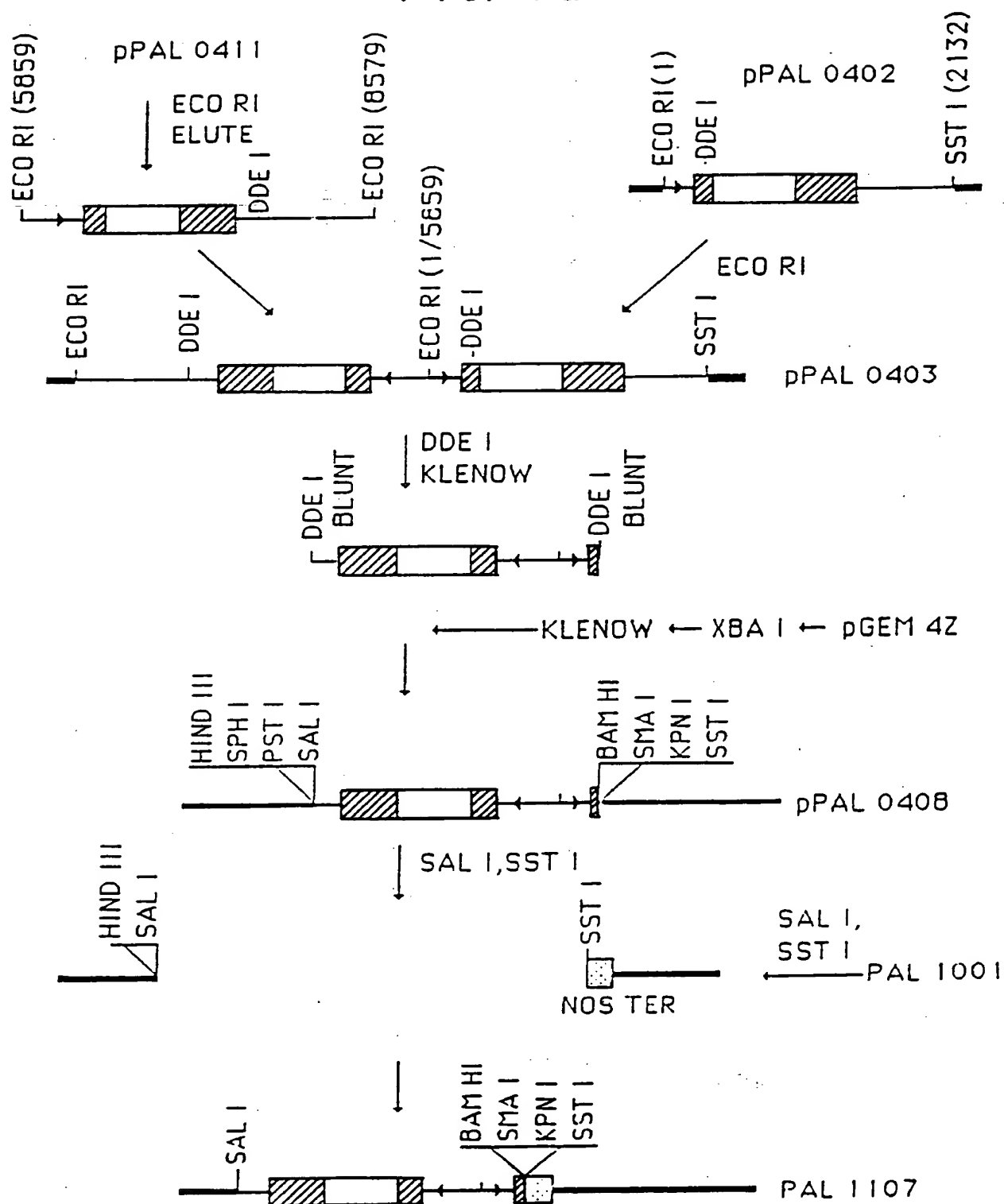
5' AAAACACACCTAAACCTGTCAACCCCTTACGCTGGAGCCTAAGTTAACT
 TTACAAAAACAATGCAACAAAGAGTTTCGATTATTCTGTTCAATTGAT
 CGATGA 3'
 stop

Eco RV

[illegible]

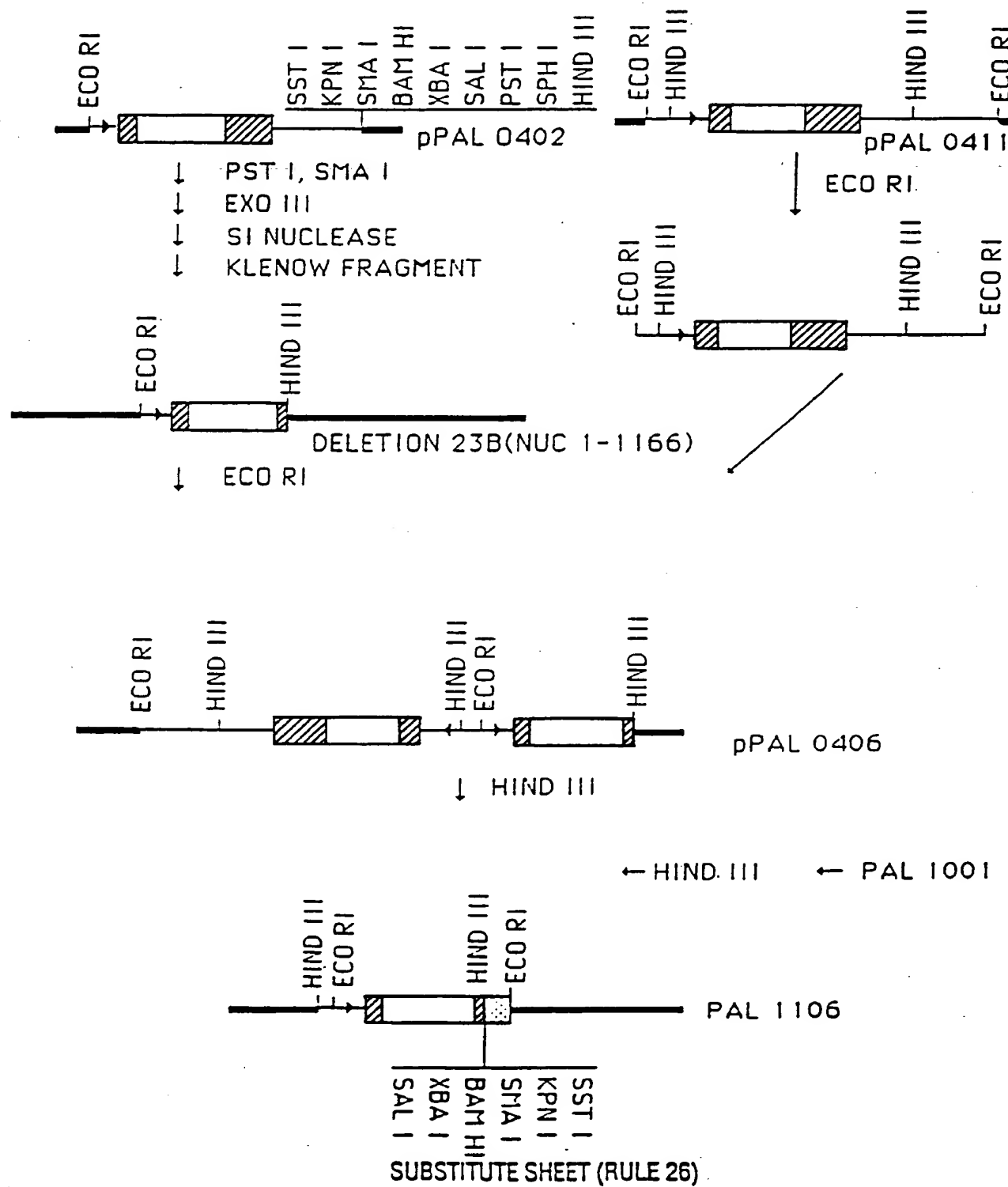
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FIG. 7a



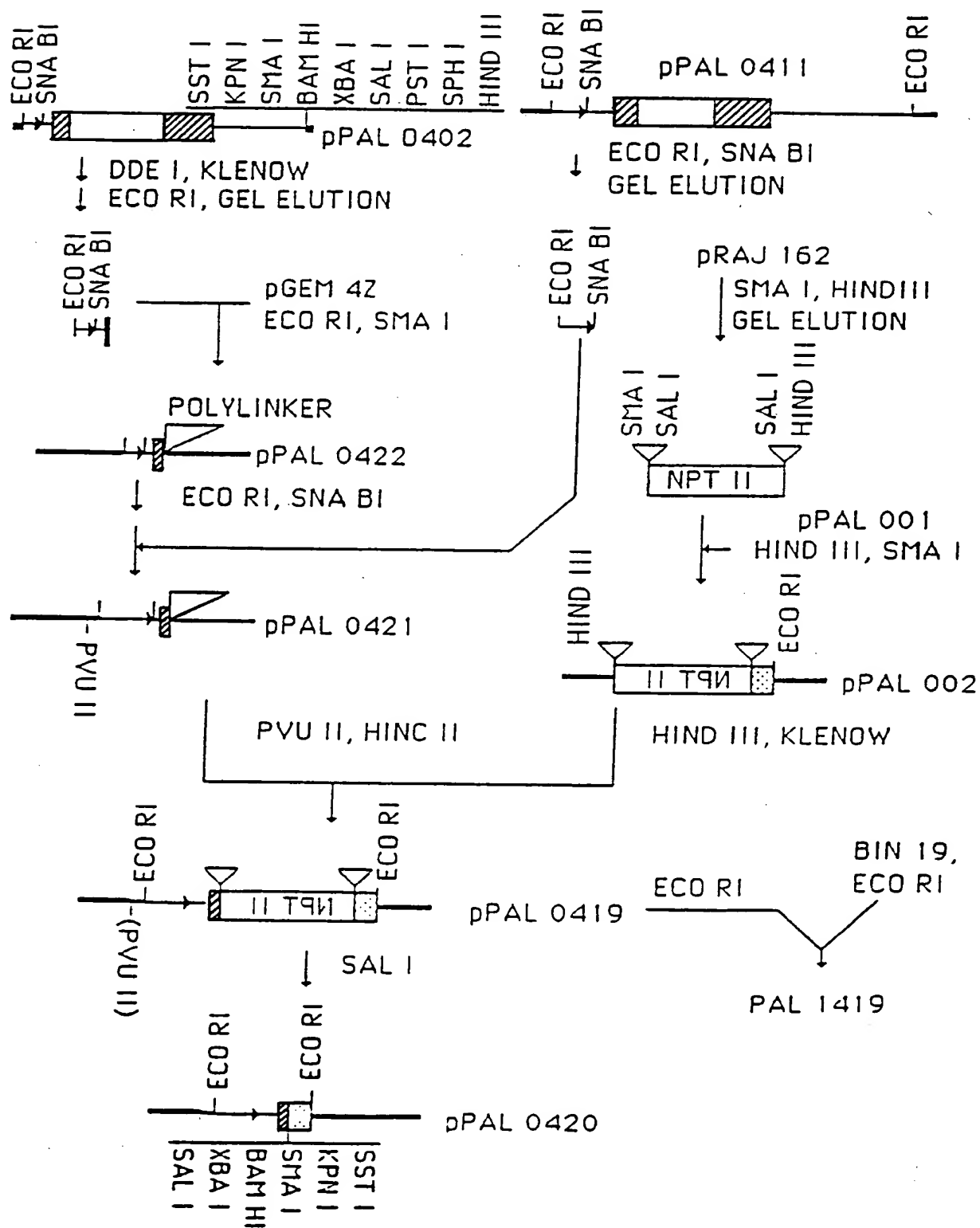
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FIG. 7b



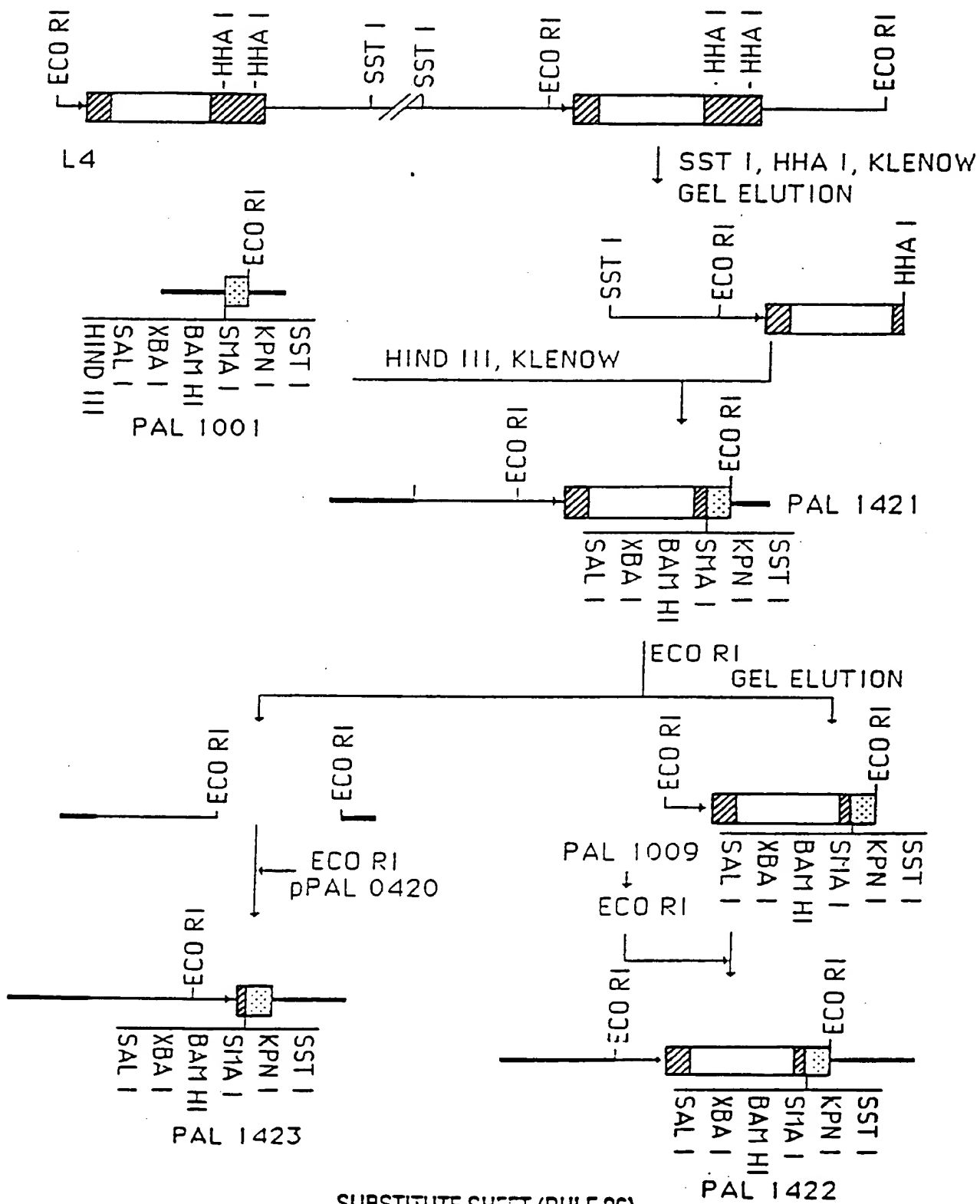
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FIG. 7c



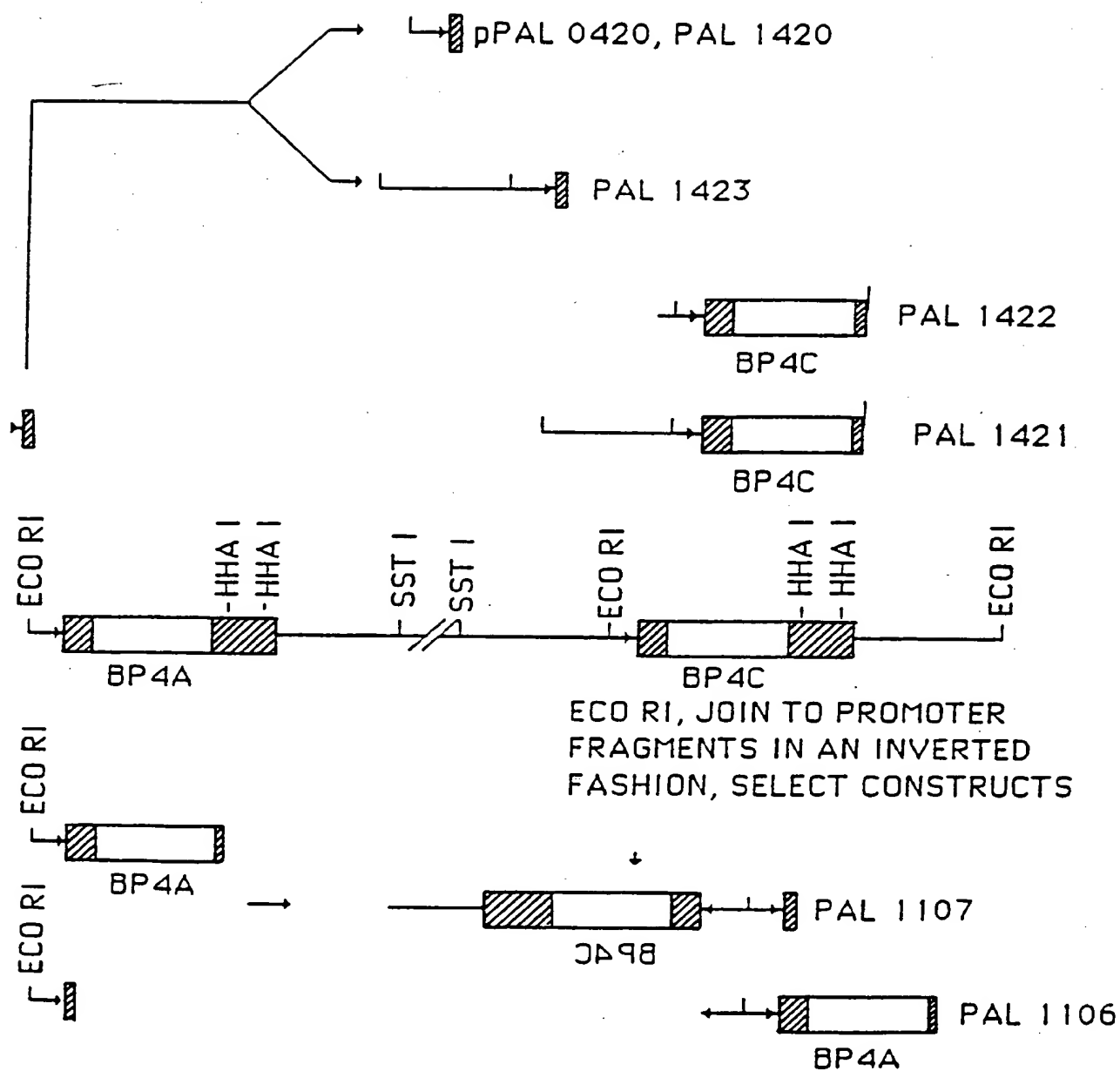
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FIG. 7d



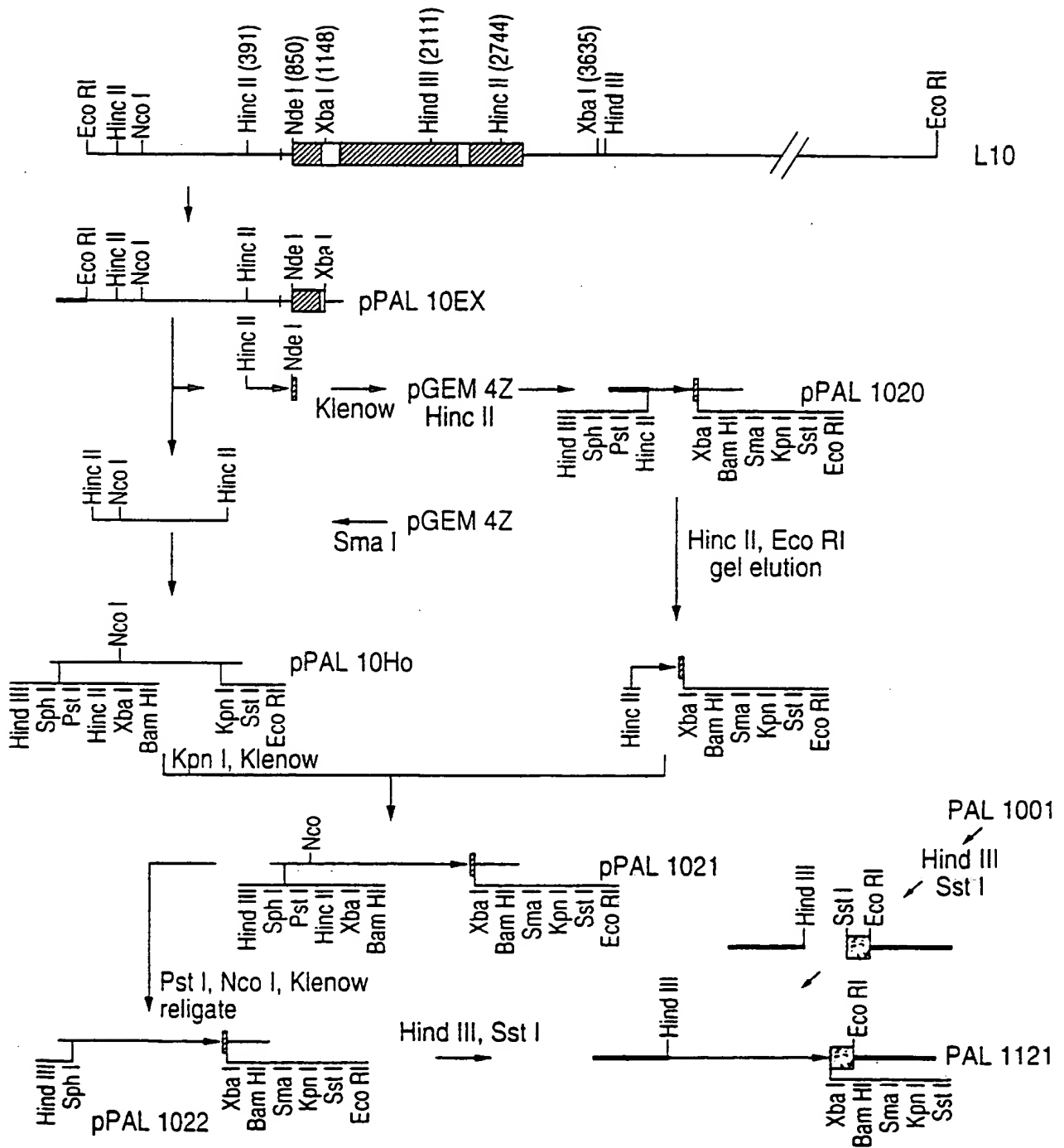
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FIG. 7e



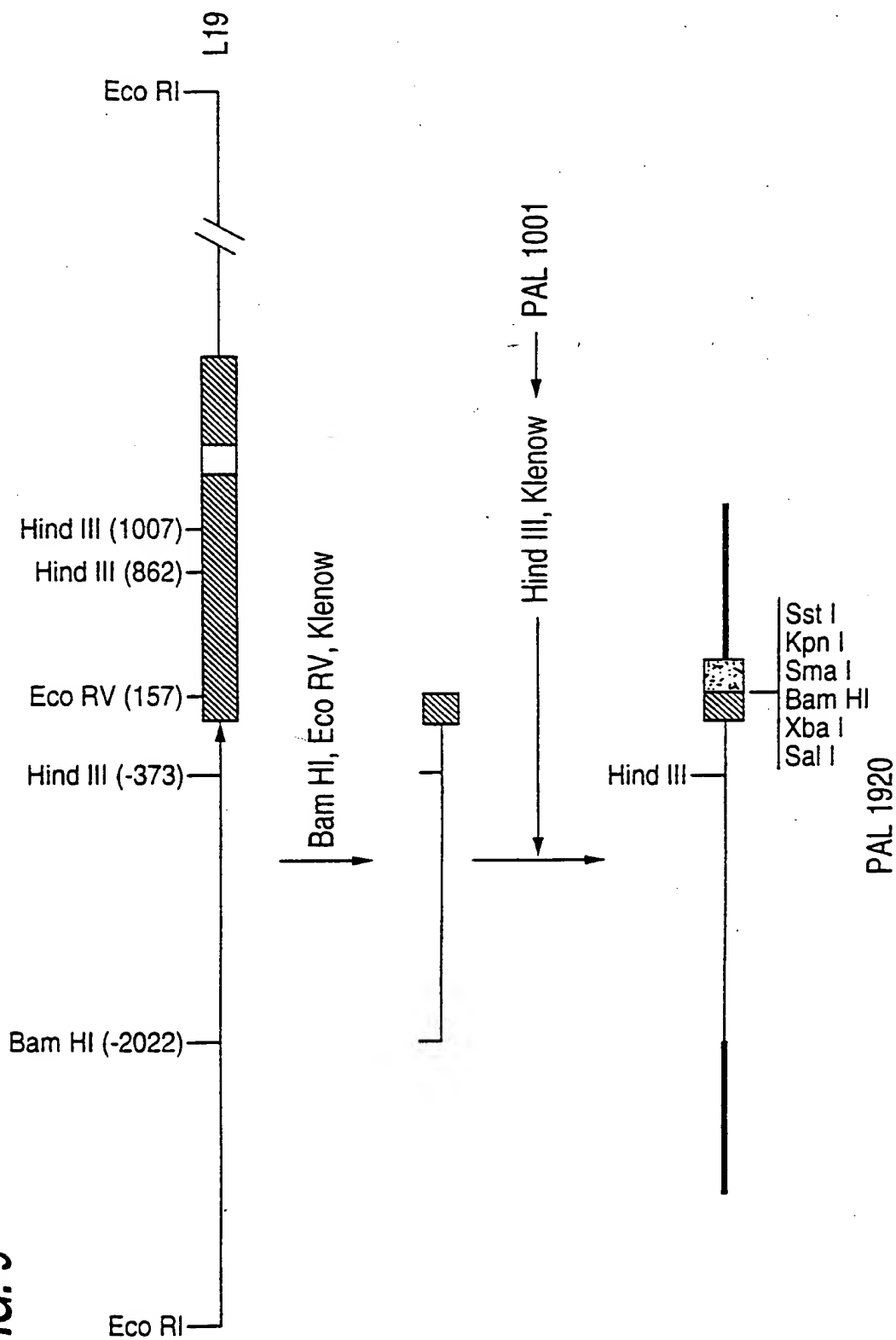
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FIG. 8



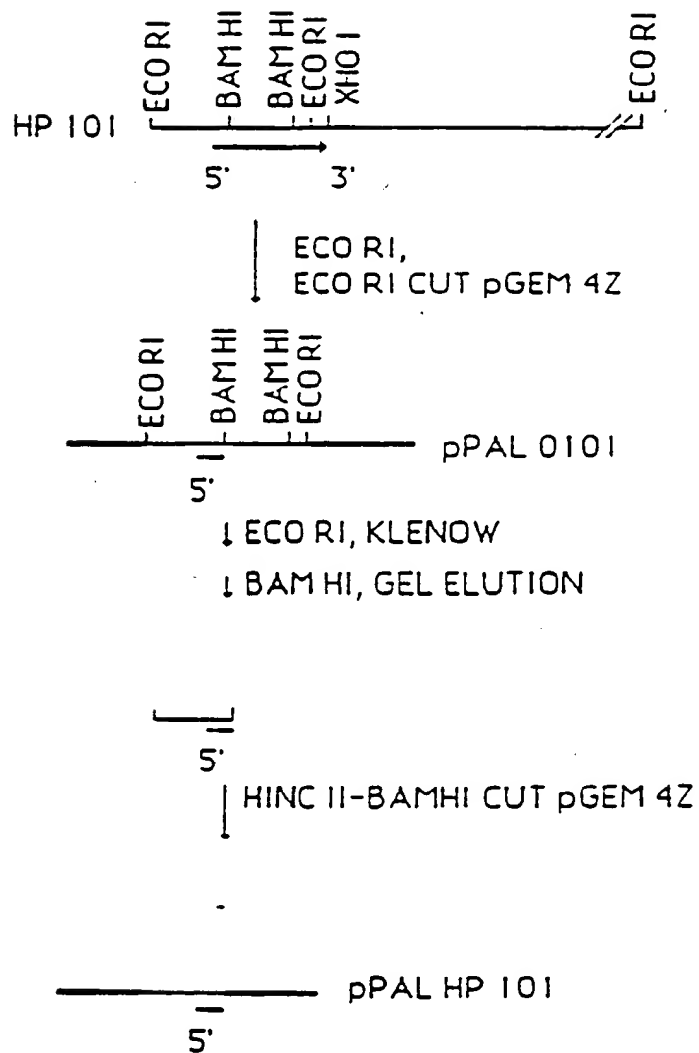
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FIG. 9



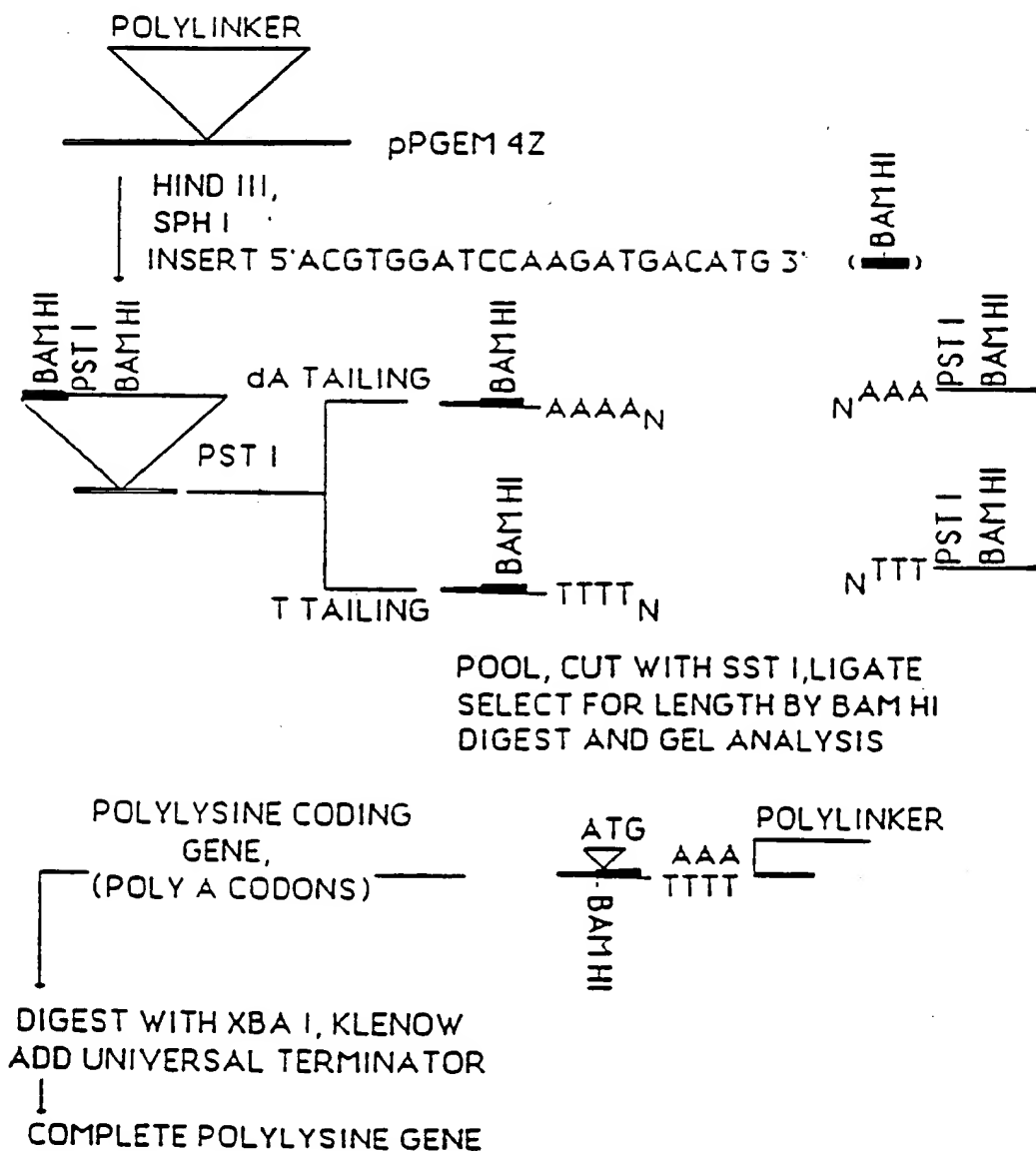
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FIG. 10



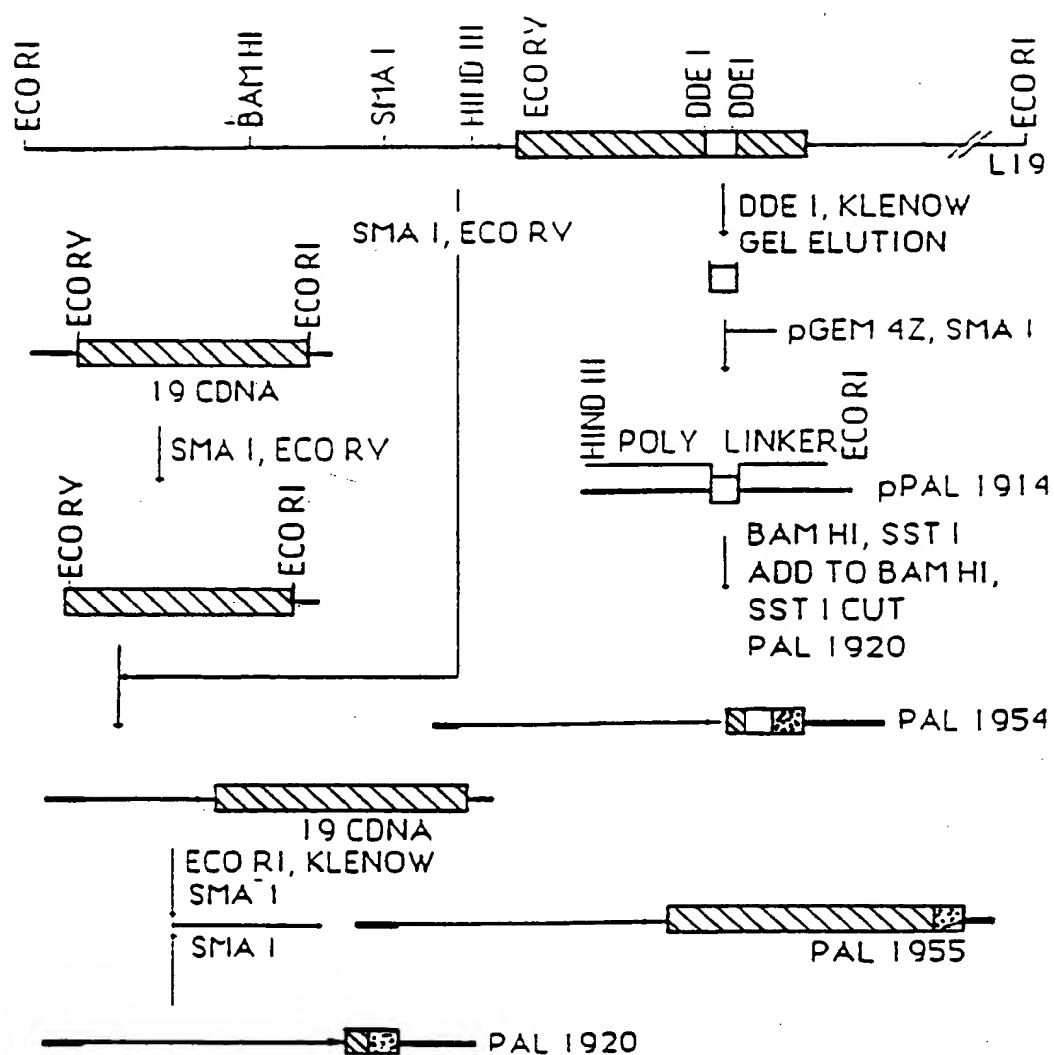
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FIG. II



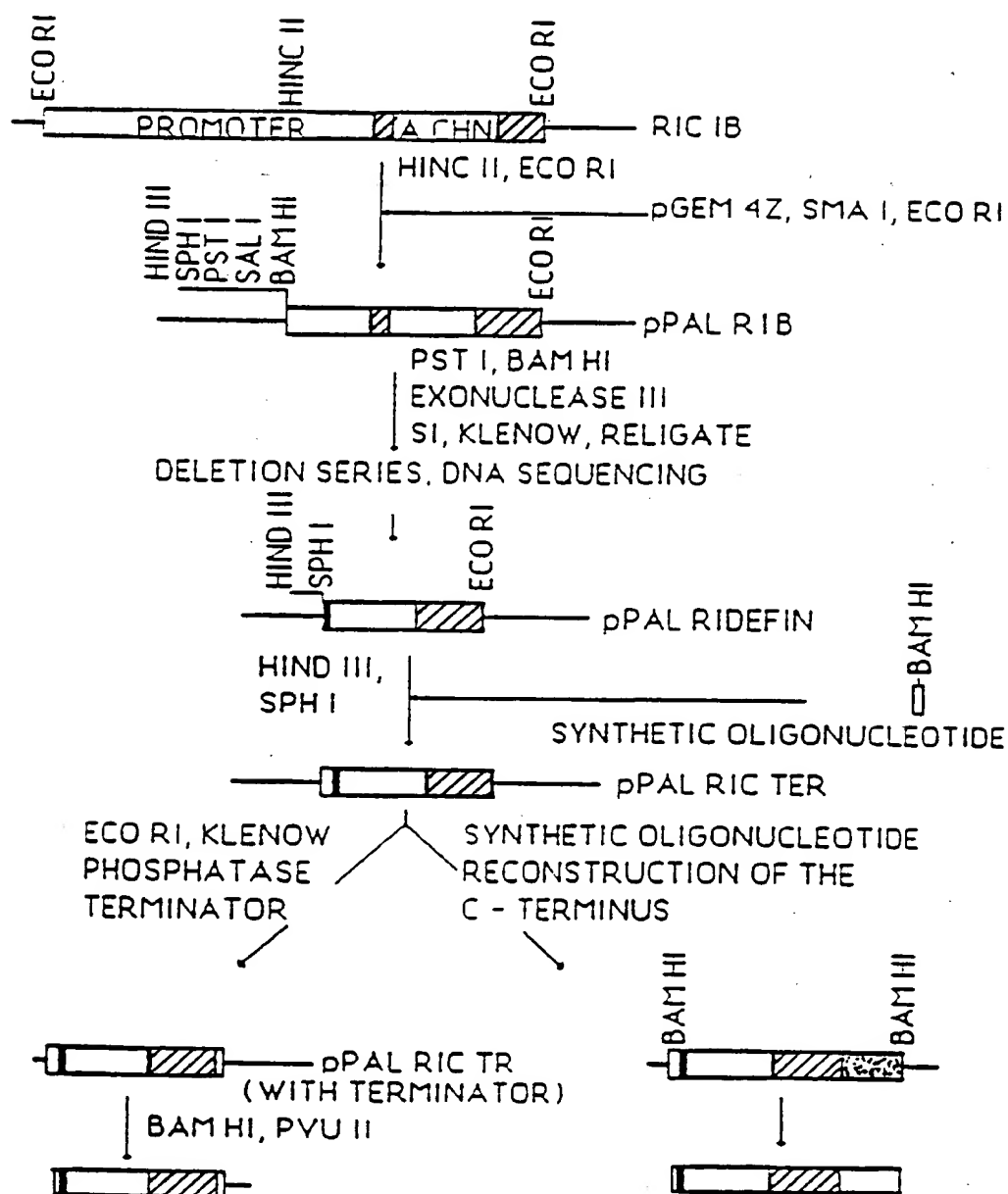
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FIG. 12



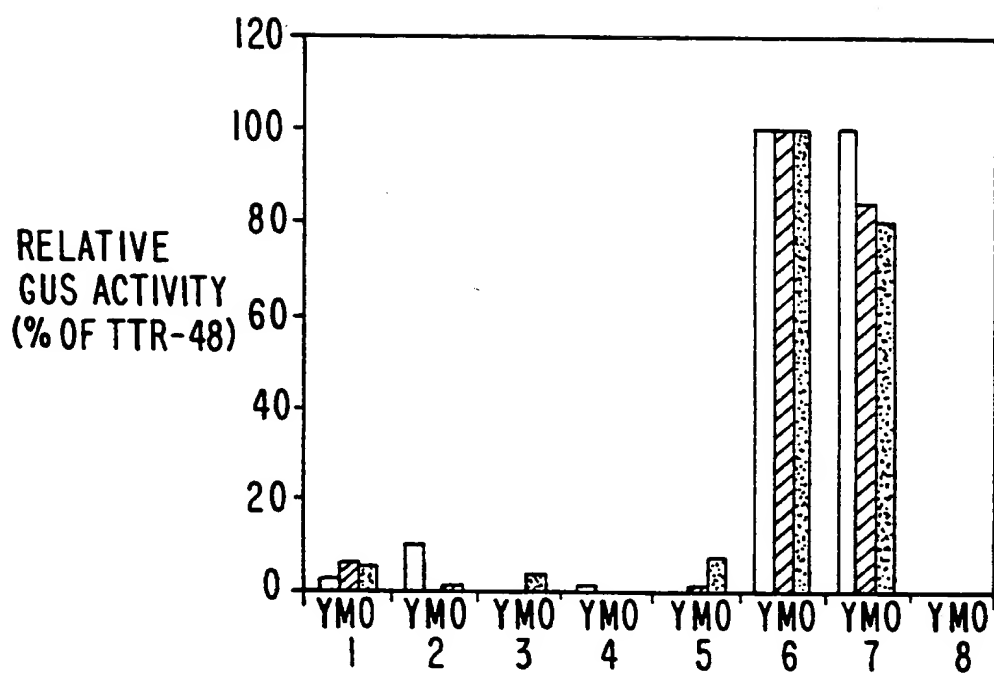
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FIG. 13



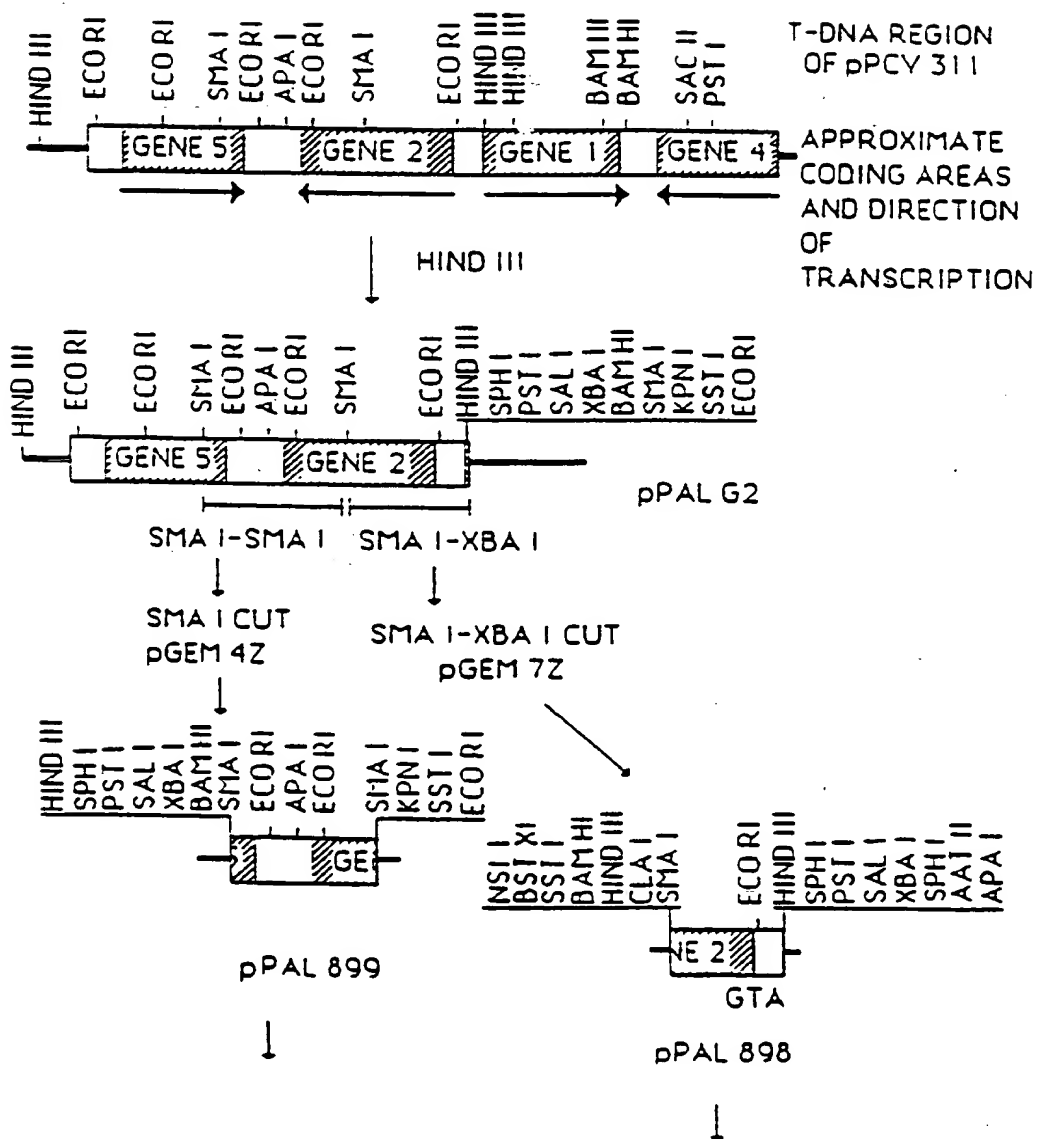
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FIG. 14



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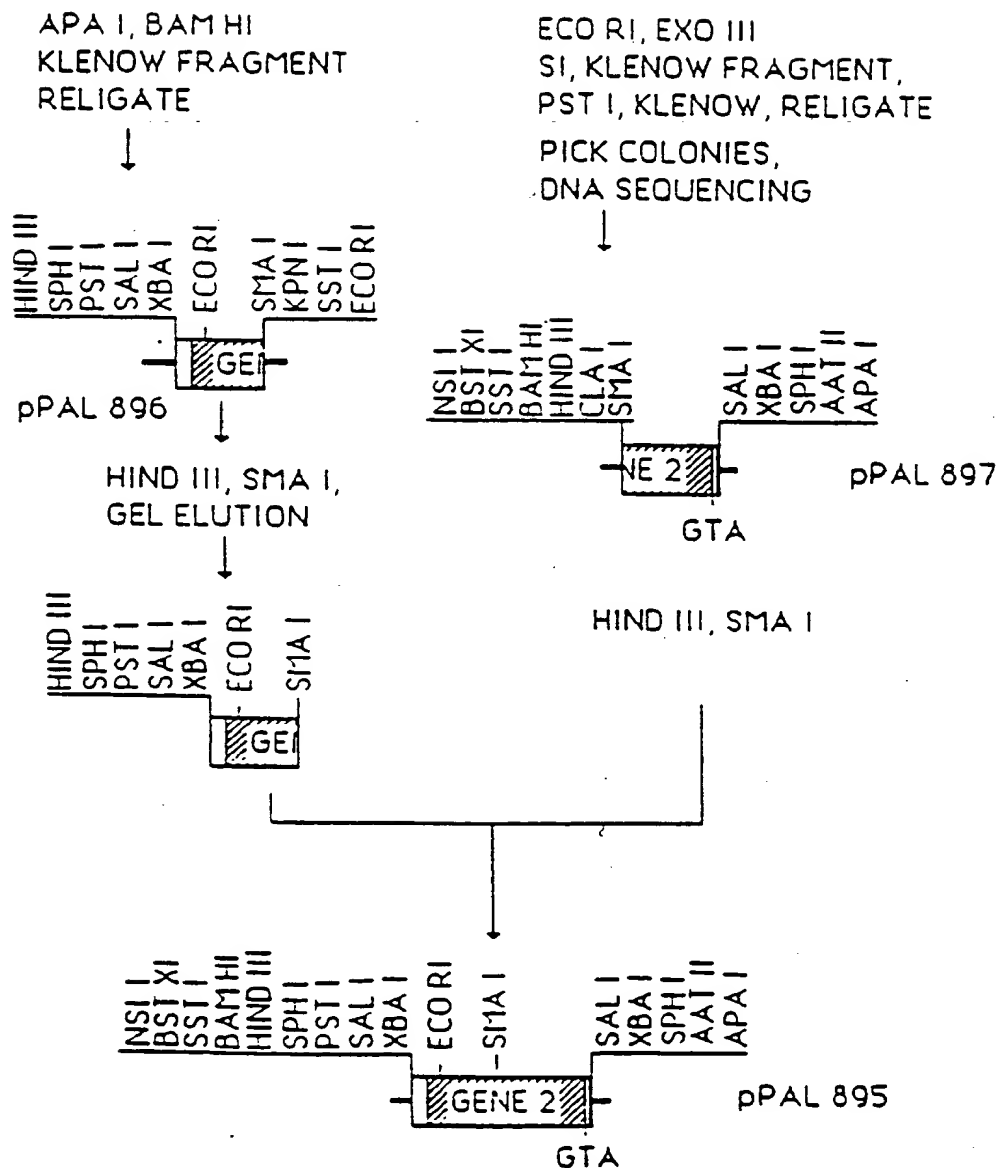
FIG. 15a



SUBSTITUTE SHEET (RULE 26)

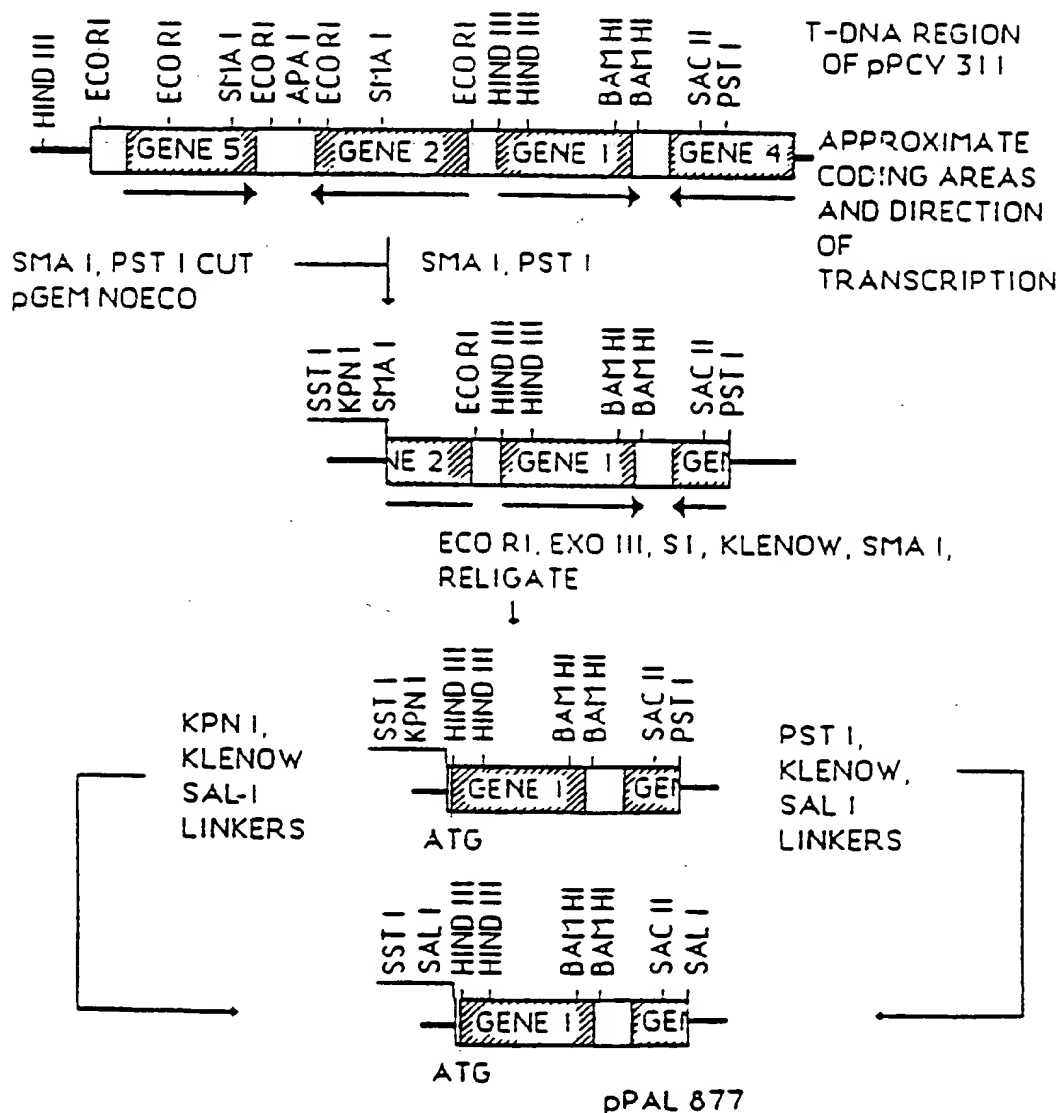
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FIG. 15b



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FIG. 16



SUBSTITUTE SHEET (RULE 26)

FIG. 17a**HYBRID SEED PRODUCTION USING BINARY
CRYPTOCYTOTOXICITY**

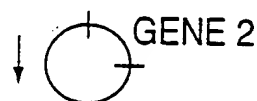
LINE A PLANT

INTEGRATE GENE 1

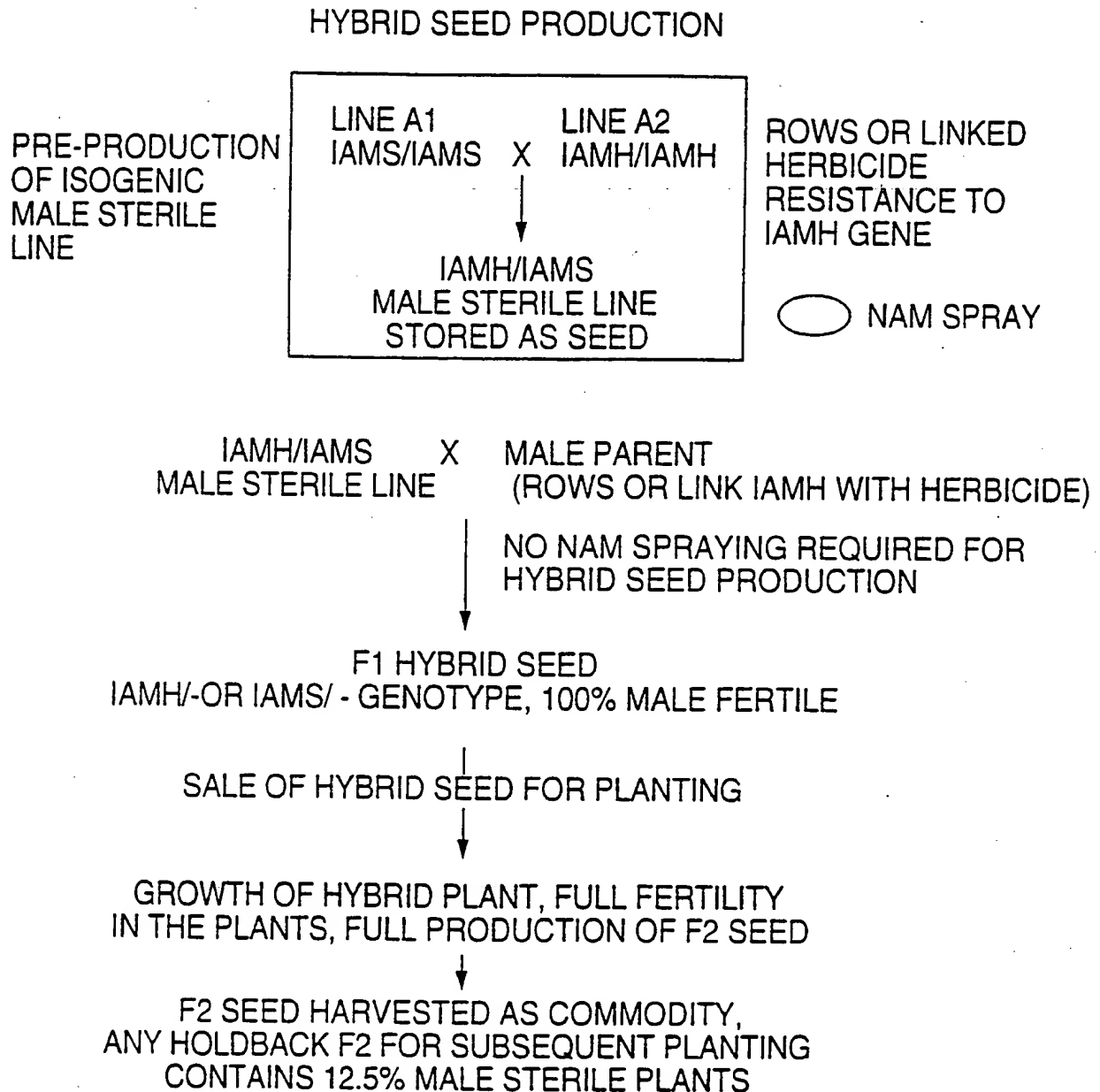
RECOVER TRANSFORMANT
WITH GENE 1 ON
CHROMOSOME Z

LINE A PLANT

INTEGRATE GENE 2

RECOVER TRANSFORMANT
WITH GENE 2 ON
CHROMOSOME ZCONVERSION TO HOMOZYGOUS LINES BY SELFING
AND SELECTION FOR THE INSERTED GENES BY
CHEMICAL RESISTANCE PHENOTYPE OR GENOTYPEMAINTAIN BY
SELFING IN ISOLATIONMAINTAIN BY
SELFING IN ISOLATIONMALE FERTILE ISOGENIC
LINE A1MALE FERTILE ISOGENIC
LINE A2

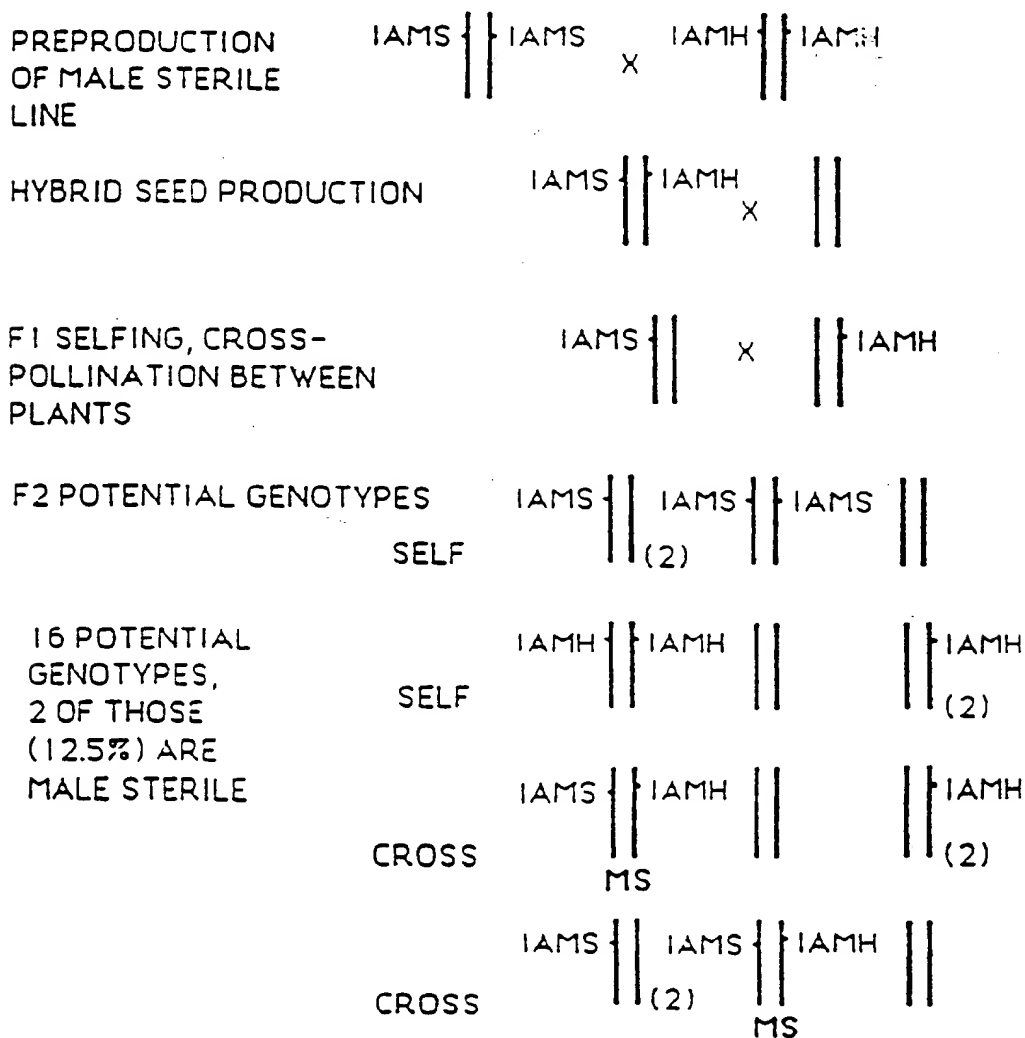
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FIG. 17b

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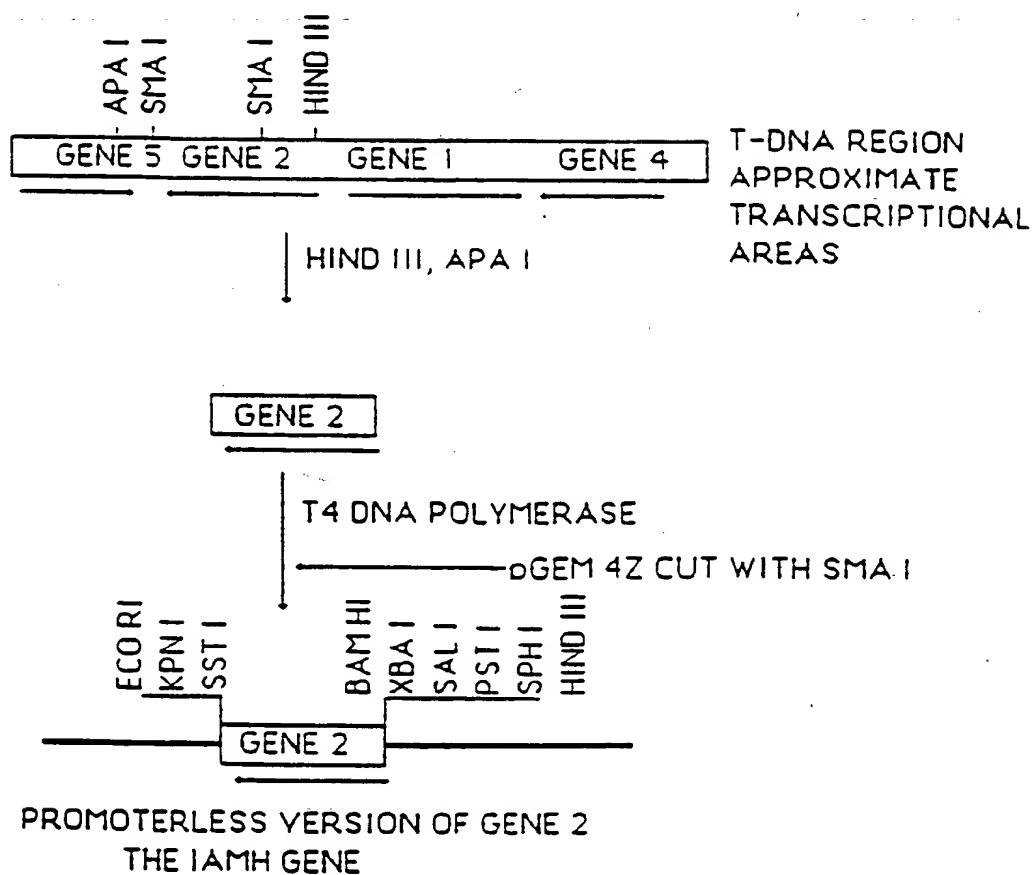
FIG. 18

SEGREGATION OF BINARY CRYPTOCYTOTOXICITY GENES IF BOTH GENES ARE LOCATED ON THE SAME CHROMOSOME OF A CHROMOSOME PAIR IN THE ISOGENIC MALE STERILE LINE

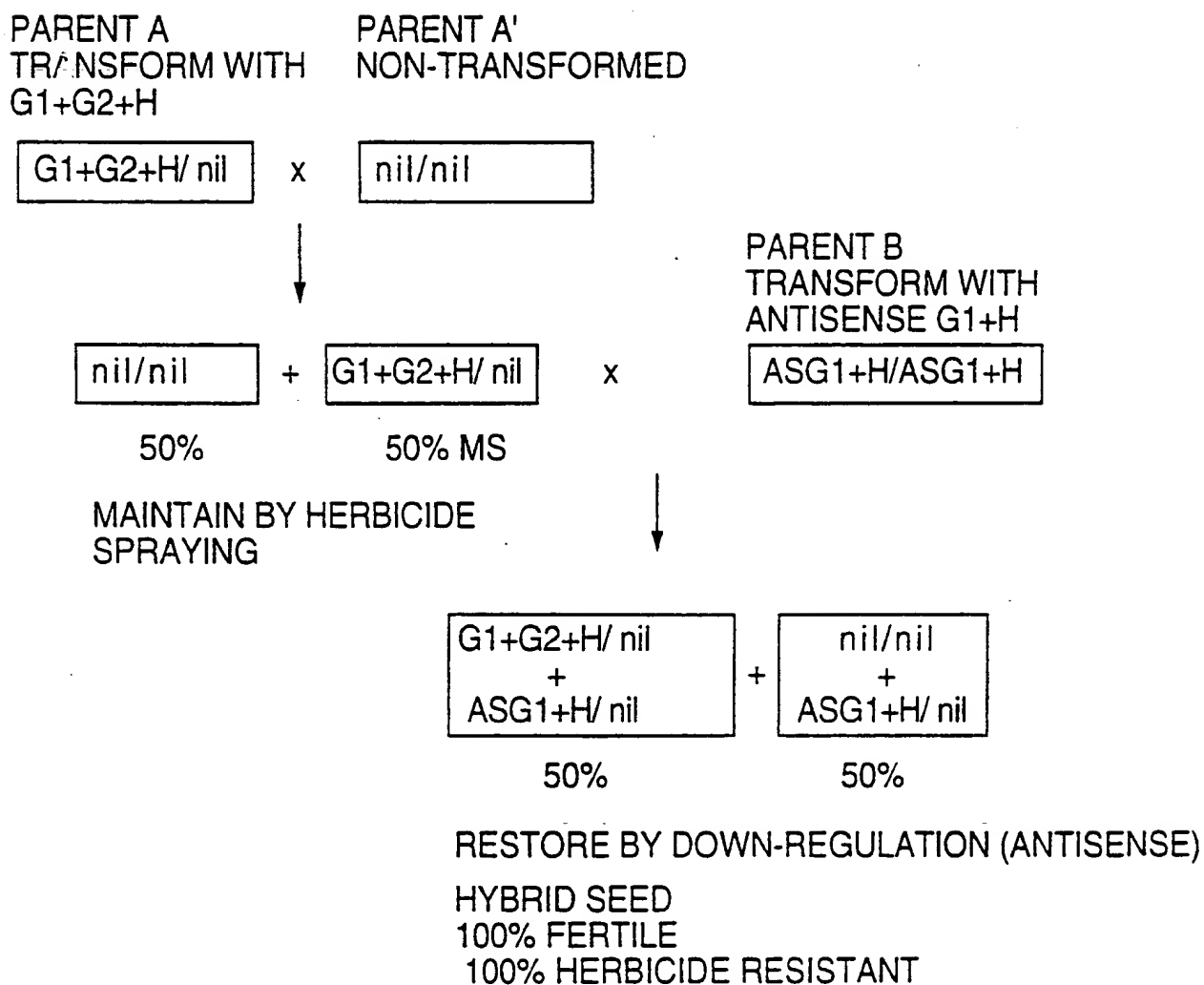


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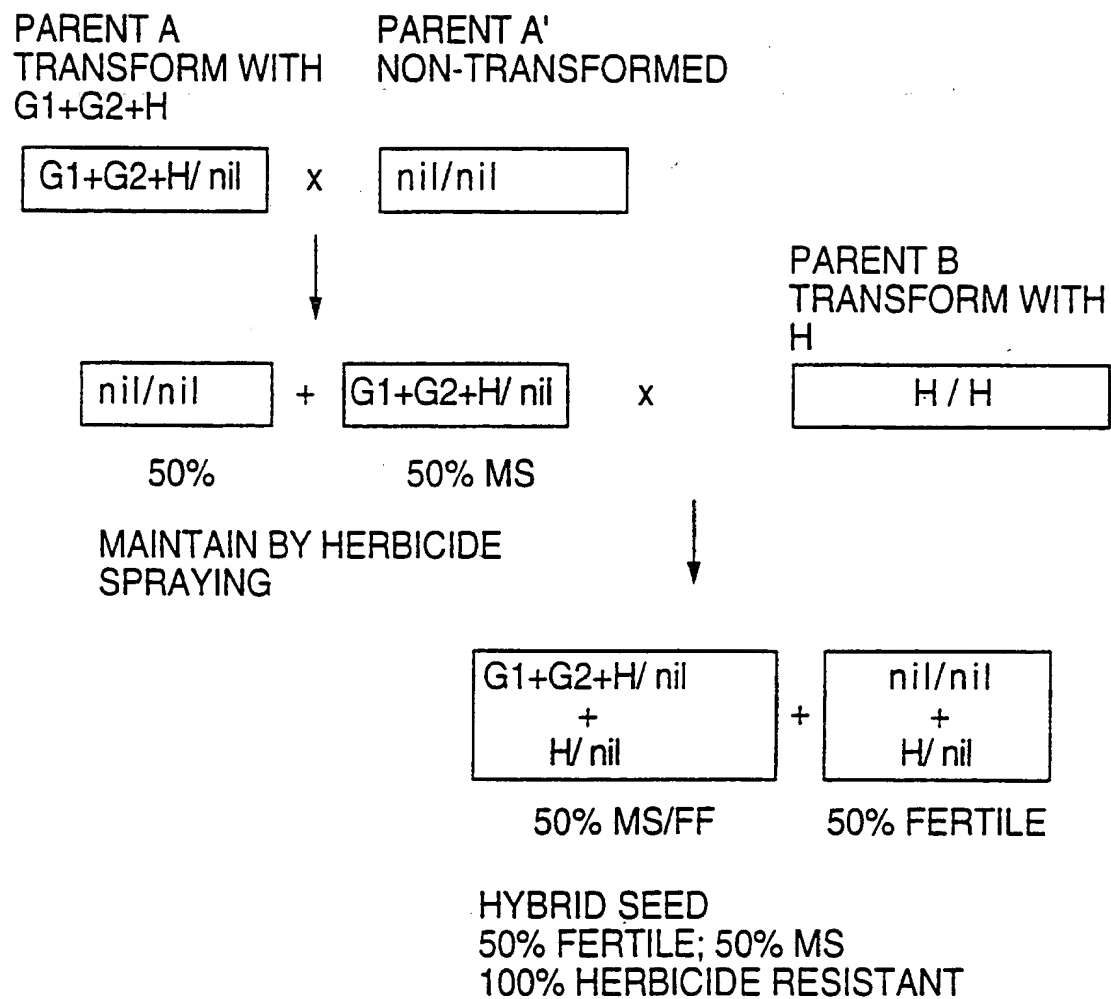
FIG. 19



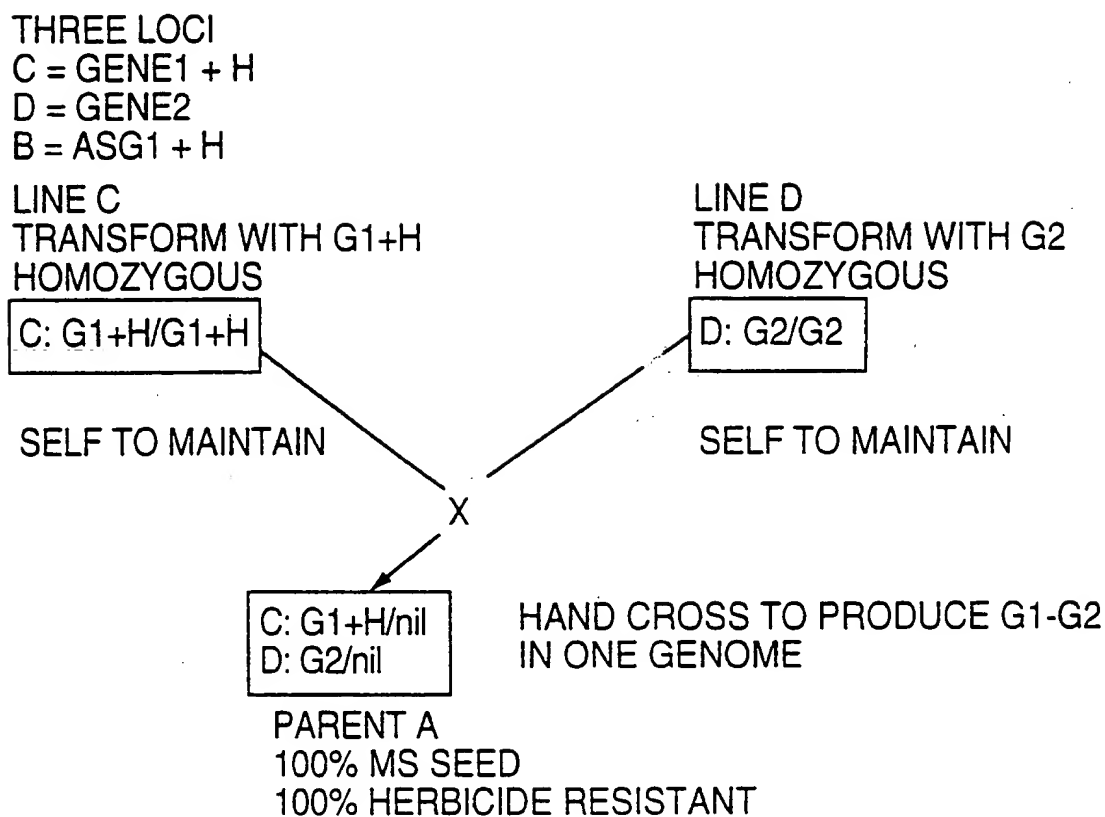
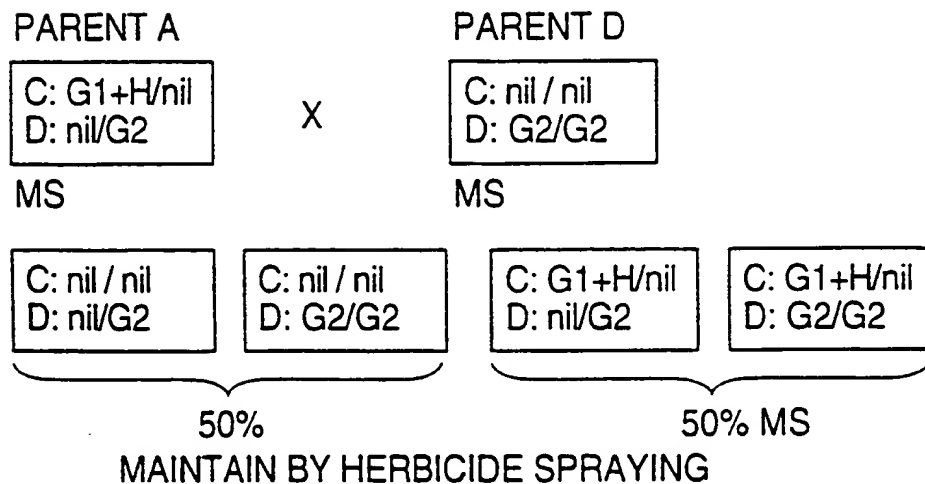
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FIG. 20

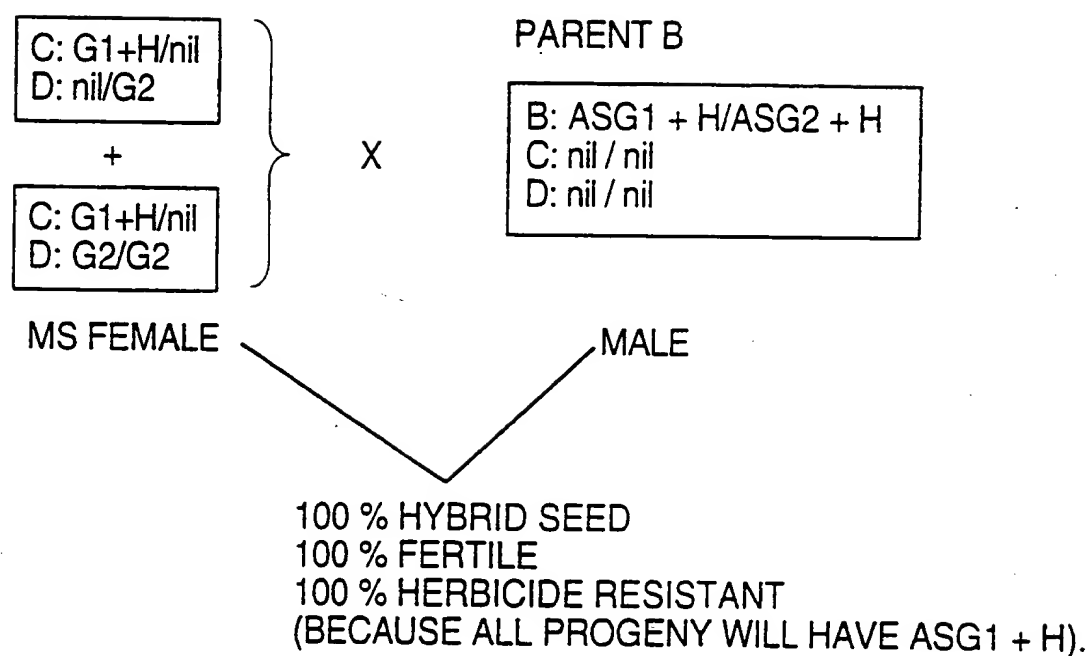
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FIG. 21

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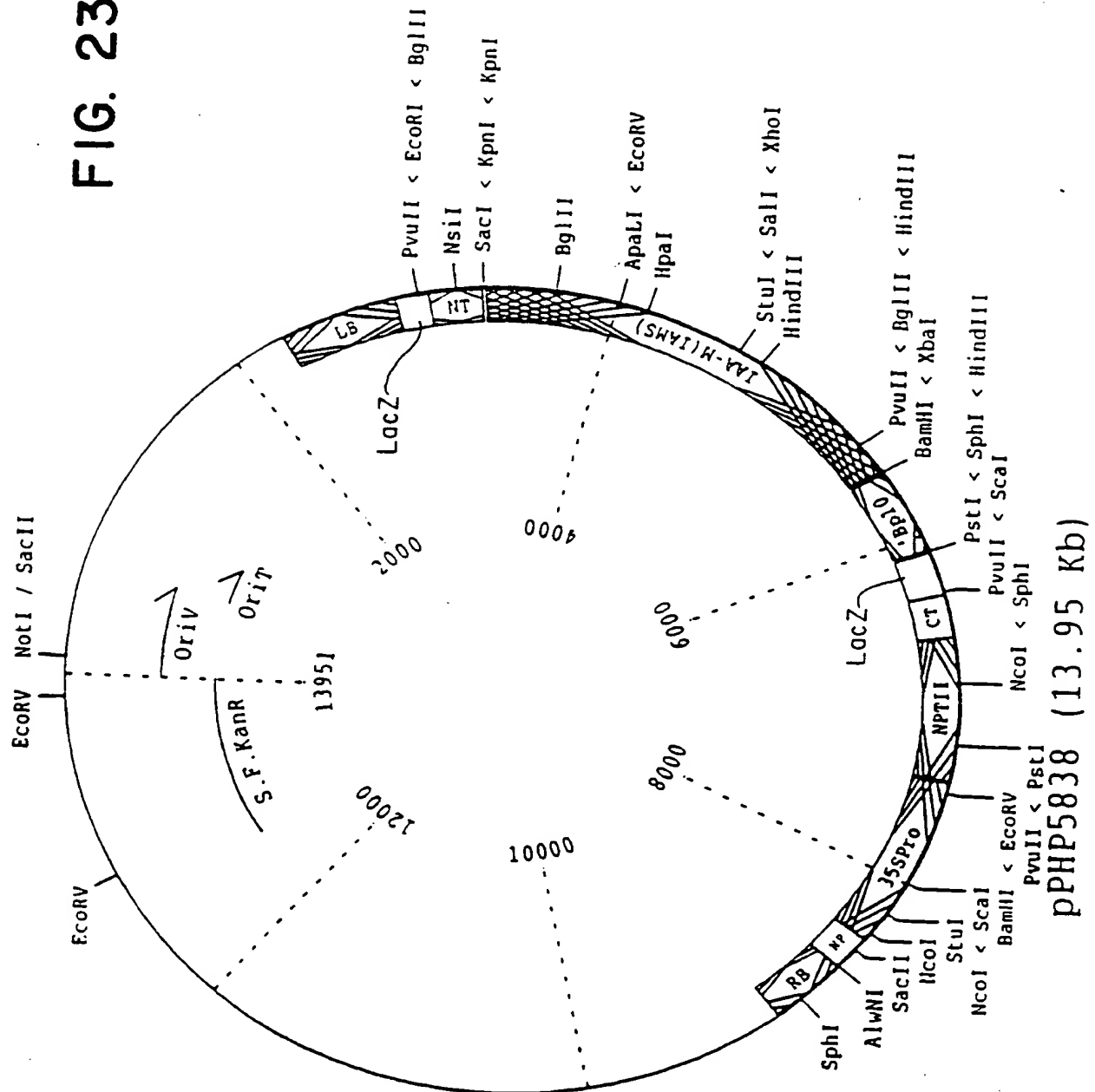
FIG. 22A**FIG. 22B**

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FIG. 22C

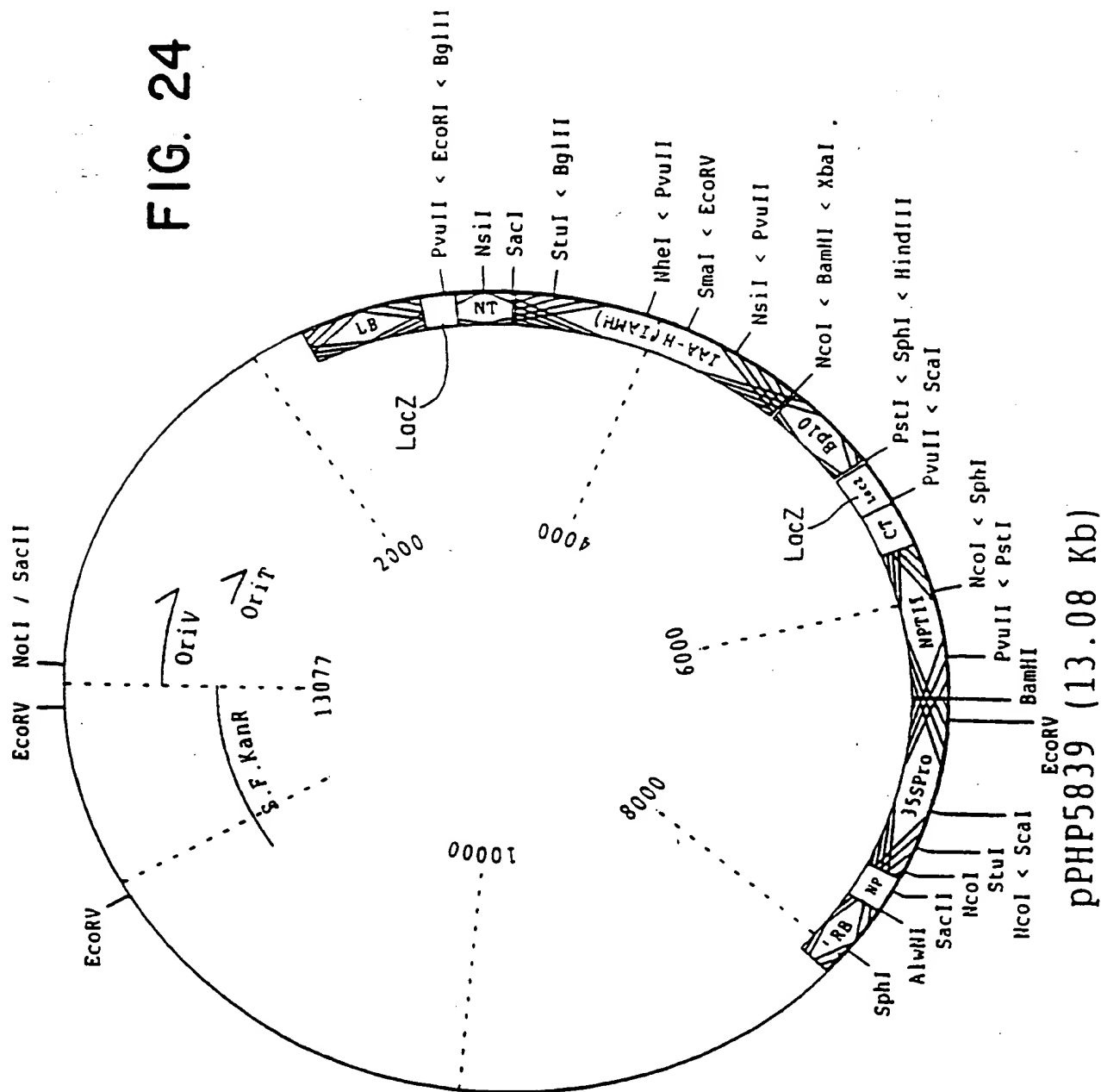
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FIG. 23



pPHP5838 (13.95 Kb)

FIG. 24



pPHP5839 (13.08 Kb)

FIG. 25

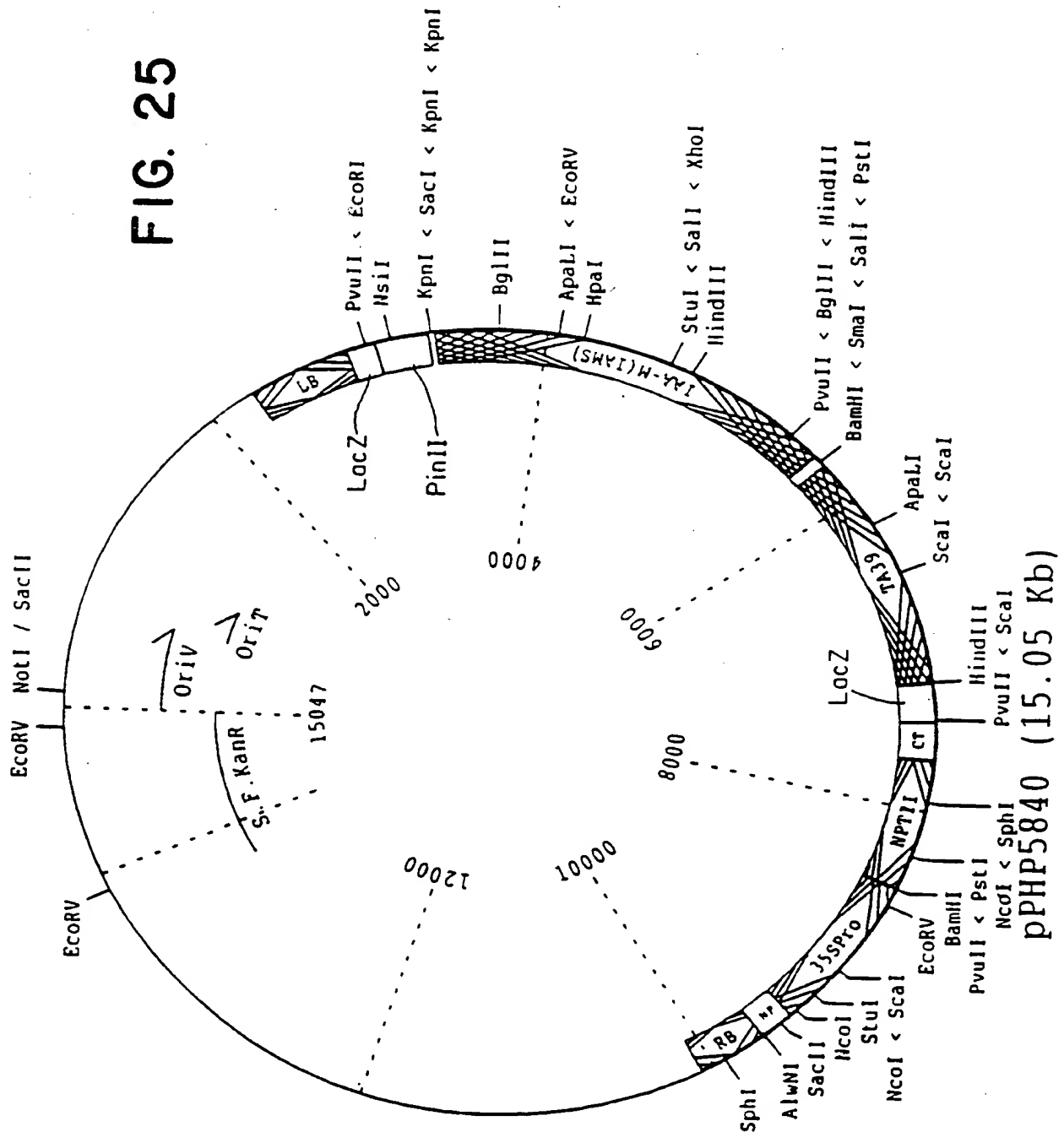
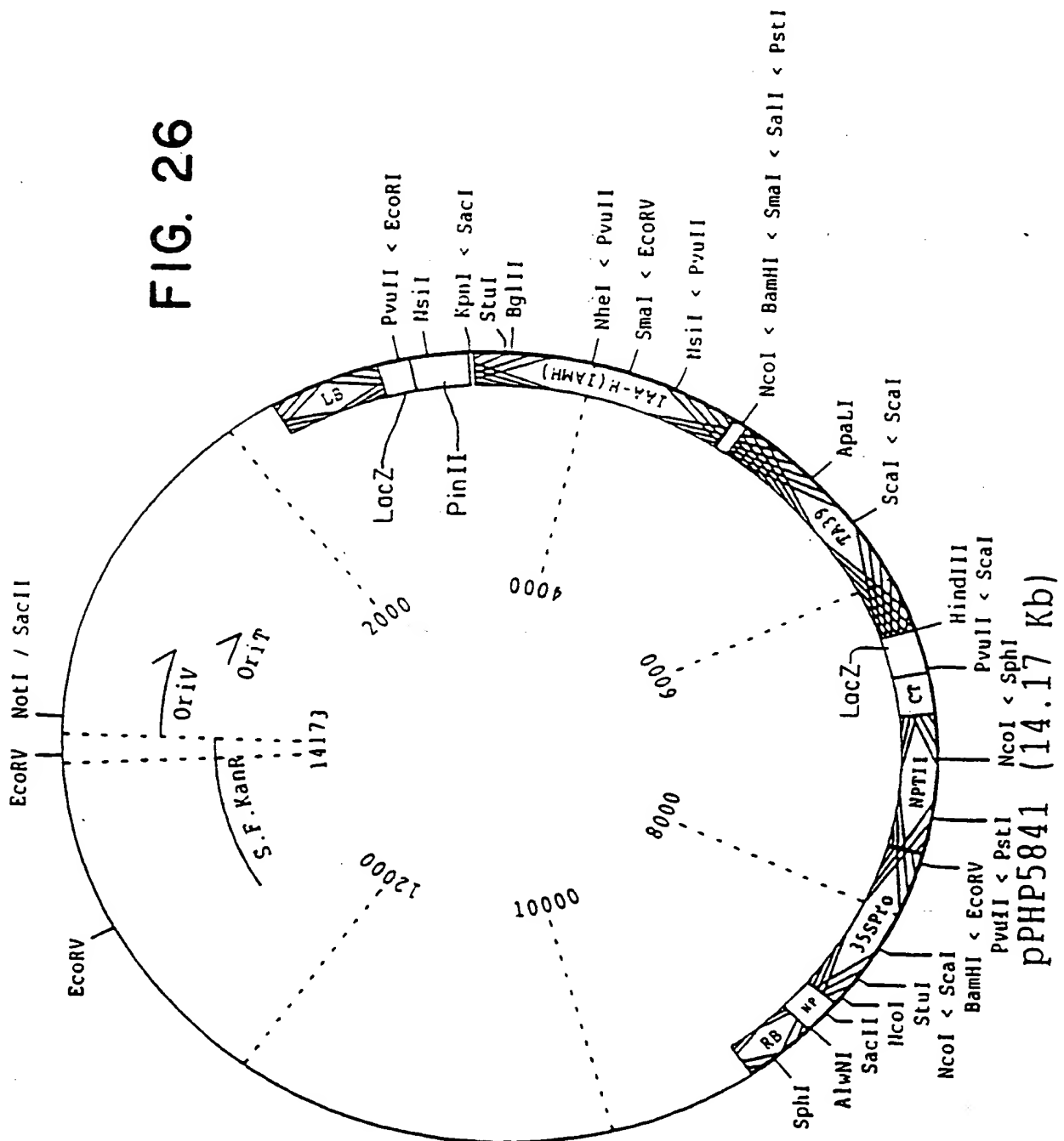


FIG. 26



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FIG. 27a

2726CTGAT GGGCTGCCTG TATCGAGTGG
2751 TGATTTTGTG CCGAGCTGCC GGTCGGGGAG CTGTTGGCTG GCTGGTGGCA
2801 GGATATATTG TGGTGTAAC AAATTGACGC TTAGACAACT TAATAACACA
2851 TTGCGGACGT TTTTAATGTA CTGCCATTCTG CCATTCAGGC TGC GCAACTG
2901 TTGGGAAGGG CGATCGGTGC GGGCCTCTTC GCTATTACGC CAGCTGGCGA
2951 AAGGGGGATG TGCTGCAAGG CGATTAAGTT GGGTAACGCC AGGGTTTTCC
3001 CAGTCACGAC GTTGTAAC GACGGCCAGT GCGaattccc agATCTAGT
3051 AACATAGATG ACACCGCGCG CGATAATTTA TCCTAGTTTG CGCGCTATAT
3101 TTTGTTTTCT ATCGCGTATT AAATGTATAA TTGCGGGACT CTAATCATAA
3151 AAACCCATCT CATAAATAAC GTCATGCATT ACATGTTAAT TATTACATGC
3201 TTAACGTAAT TCAACAGAAA TTATATGATA ATCATCGCAA GACCGGCAAC
3251 AGGATTCAAT CTTAAGAAAC TTTATTGCCA AATGTTTGAA CGATCTTGCA
3301 CGTTGAGCTC GGTACCCGAT CCGTAGATTA ATTTCTATTG CGGTAGTTAT
3351 ATCTCTTCCA AGAGTGTTCTG AGAGGATTGT CCTTTGCCAA AATACCTCCA
3401 CAATTGTGGA TAATTGCACA GACGGCGTTA CACGCGGTCT GAATAGCGCC
3451 CTCCACCCAT CCACCGGTGA AGGAACAAC GCAACCCGCC AAGTAAACTC
3501 CGGTATCATT AGTCATGTCC AGCGCTTGAA AGAAAAGTTC TTCAGAATAA
3551 AAATCCTCGC CACGCCGGTT GAGTTTGAAA GCTCCCCCGG CATTCTCGTC
3601 TGTAAGCCAA TCATGTTGAA CAACATTTTG GTCGTAATCA GCGCAGGCGG
3651 GAACTAGATG CTGGGCAAAC GCCGGGAAAG ATCTCGAAAT TGCGTCCCCG
3701 AGCAGACAGA ATCGCTCTTT TTTGTCGGGA ACCGCCAACA GCTTGTGGGA
3751 GTCGTCCTCC CATGTATAAC TGATGAGCAC CAGACCTTTA CCATTCGGgT
3801 CCTGgGGCTC ATAGTCCAGG CAGTACACTG CTTTTGCGAT CCCGTCCATG
3851 AGGACACAGG ACGGGAGGAT ATGGTCTAAC CAAAATTTTC GTTCAGTCAG
3901 CAGGAAGAGT TTTGACGAGC CTGTCATATG GCTGTTATCA ACCGCTTGGT
3951 TCACTGGTGC ACGAAAAATG GTGGTATCGC ATGTCAGACA ATGCCTGAGT
4001 TGGATATTTG CGAGTCCAGA TGTAACCACC ACCTTATCAT AAAGTTCAGA
4051 TATCCCGCTC TTAAGCCTTA TCTTTATTTT TGTCTTTTCT TTCTCAATCG

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FIG. 27b

4101 CCCTGACTTG AACATGGCGT ATACGCTGGC TTACAGACAC ACCGTTAACC
4151 ACTTGAGTGG CTATTCGACG TGGAAGTTCT GAGATTCCTT CAGAGCACAT
4201 CCGCTGATTT TCTTCATATC CGTTTATGAC CAAGCGAAGG ATCTCAATAA
4251 ACCCGCTTTC AAAAAGTGA CCAAACCCGC CAGATCCTAT TCCCATTAGC
4301 TTGAATAGGT CCCAATCATG AGGGAACTC CATGTTTCAC CACCAGGAGG
4351 ATGCGTGCCC AGAAAGATCC TCTCTATCGC TGAAGAGAAG GACTCCCTCC
4401 CGAAACGGTT CAGCCAAGTT TGCCAGGAGT CATGAGCCCG CCTAATGTCT
4451 CCTGATTTCA AGGCTTGAGT AATAGCAACA GGCGAAGCCA ACACAATATC
4501 TCCCTCATGG AAACCGTCCT TCAAGAACGC ACGCCAACCG CTGTAAACGC
4551 GATGGAACAG CTTCGGTGGC TGCTGCCCCG CTTTCCACAT GTATCGGAGG
4601 CCTTGGTAGA CCAAGTTAGT GTCGACTGTG CCGGGATTG GGAACGGCCT
4651 CATCGAAGAC AGGCCGTACC GCTCGAGGAA GAAAAACAAG CACGATGCAG
4701 CAGGAGGAAA TCGCATCGCC CCCATTTCGG CCACCACGCT GGGAGCGTCC
4751 TTGAAAGCAT GTGACCAAAG CTTGCCTCCA ACCCGATCAC TTGCTTCATA
4801 TATTGTAACA TCGTCTACAC CAGCATGAAG CAGTTCGCTT GCCACCACGA
4851 GTCCGGAAAT GCCAGCGCCA ATGATCGCCA CTTTGGCTT AGGAACATCT
4901 TCCGGAAAGA AGCCGATCCG TCCACTATCG GAACATTGGT CAAAAACGA
4951 TCTGTAGTCA TAGAGCAAGT CAATTGTTGG AAAGGAACCT GCCGAAGCCT
5001 TCAGCATCAG GTTTCGACCA TAAGCTACCA TGTCAAATGG TACGGCCTCT
5051 TCAAGGGTCA GGGTAAACCC GGTAACGCGT TTCGTCGTGC CTTTCTCGTA
5101 AGGCGGCATT TGCAGGGTTC GGGAGCGGGC AGTCTTAGAG ATTGAAATGG
5151 CCACGAAATG ACTAAGATCG CTGGATGCAC ATCTTTTACC GCTCAGCAAA
5201 AACAGGGTTT GGAGAAGTTG TGCTTCGGAG AAATCCATAC AAAATGGTGC
5251 AAGGTCGATG GCAACGAGAC CACTCACTCT TGCTCGCGCC CAAGGTTCCG
5301 ATTCAAGTAT CCGCCCCAGA ATTCCTTGC CAACATAGAT GTAAGCGGAG
5351 AGGGCTGCTA CCTTCTCACC AGCTGAGATC TCGGGAAAGC GACCGTCGGC
5401 CAGCCTTTTG CAAGCTAACC CAGCGCTGCA CTCGCCGGAG ATTTGAGTAA
5451 TCCTCCTTCC CCTAGAAGCT TCACGTTCTG AGAAGGCATC GGAAACCCTG

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FIG. 27c

5501 CGGTCCAATT CATCAGCCTT ATCGACCATT ATCAGATCCA CCATTTTGGT
5551 AGAGAAATGA TCCCACTGGT TATCAAGGAG AGCTGAAGCT GACATgGtGG
5601 ATCCTCTAGA GTCTATGTGT TTTTGTGGGG TATTAATGTT TTATTTTAT
5651 TTGGTTATGT TTCGGGGTTT GAATTTCGAG TTGTGAAAAT GCTTGAGGAC
5701 GTTTTTATAG GGTTTTTTAA GACTGCTTTT TCTGATTTTC TTTGGCATCT
5751 CCTCTCATTT CGCTATTCAT TTCCATATTT GGGAACATTG AAGTTTAACC
5801 TCTCGCTATT ATTAGTGTTT TTAGTTTCTC TTAACATTTT TCTCCCTTTT
5851 CTAATTTTTG GCTTTGGTTT CCATGTCTTT GAGAATTTGG CTATTTTGA
5901 TTTTAAAAGA ATACTCTGAG AGAGAAAAAT CTATTAGTCA CGTGAATAAG
5951 TTATAGACAT AAACCTGAAT GTATTTCAAG AAGTTCACAT GCAAACCTGAT
6001 AAATTTACAG AACTATTATT ATTATTATTA TTATTATTAT TAGTTTTTTT
6051 GGTTAAACAC TCTTTTTTTT TGTCAGACCT GCAGGCATGC AAGCTTGGCG
6101 TAATCATGGT CATAGCTGTT TCCTGTGTGA AATTGTTATC CGCTCACAAT
6151 TCCACACAAC ATACGAGCCG GAAGCATAAA GTGTAAAGCC TGGGGTGCCT
6201 AATGAGTGAG CTAACTCACA TTAATTGCGT TGGGCTCACT GCCCGCTTTC
6251 CAGTCGGGAA ACCTGTCGTG CCAGCTGCAT TAATGAATCG GCCAACGCGC
6301 GGGGAGAGGC GGTTTGCGTA TTGGGCGCTA TCGGGGGATC TATAGGGACT
6351 TTAGGTGATC TGGATTTTAG TACTGGATTT TGGTTTTAGG AATTAGAAAT
6401 TTTATTGATA GAAGTATTTT ACAAATACAA ATACATACTA AGGGTTTCTT
6451 ATATGCTCAA CACGTGAGCG AAACCCTATA AGAACCTAA TTCCCTTATC
6501 TGGGAACTAC TCACACATVT ATTATGGAGA AAATAGAGAG AGATAGATTT
6551 GTAGAGAGAG ACTGGTGATT TCGAACCCCA GAGTCCCGCT CAGAAGAACT
6601 CGTCAAGAAG GCGATAGAAG GCGATGCGCT GCGAATCGGG AGCGGCGATA
6651 CCGTAAAGCA CGAGGAAGCG GTCAGCCCAT TCGCCGCCAA GCTCTTCAGC
6701 AATATCACGG GTAGCCAACG CTATGTCCTG ATAGCGGTCC GCCACACCCA
6751 GCCGGCCACA GTCGATGAAT CCAGAAAAGC GGCCATTTTC CACCATGATA
6801 TTCGGCAAGC AGGCATCGCC ATGGGTCACG ACGAGATCCT CGCCGTCGGG
6851 CATGCGCGCC TTGAGCCTGG CGAACAGTTC GGCTGGCGCG AGCCCCTGAT

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FIG. 27d

6901 GCTCTTCGTC CAGATCATCC TGATCGACAA GACCGGCTTC CATCCGAGTA
6951 CGTGCTCGCT CGATGCGATG TTTCGCTTGG TGGTCGAATG GGCAGGTAGC
7001 CGGATCAAGC GTATGCAGCC GCCGCATTGC ATCAGCCATG ATGGATACTT
7051 TCTCGGCAGG AGCAAGGTGA GATGACAGGA GATCCTGCCC CGGCACTTCG
7101 CCCAATAGCA GCCAGTCCCT TCCCGCTTCA GTGACAACGT CGAGCACAGC
7151 TGCGCAAGGA ACGCCCGTCG TGGCCAGCCA CGATAGCCGC GCTGCCTCGT
7201 CCTGCAGTTC ATTCAGGGCA CCGGACAGGT CGGTCTTGAC AAAAAGAACC
7251 GGGCGCCCCT GCGCTGACAG CCGGAACACG GCGGCATCAG AGCAGCCGAT
7301 TGTCTGTTGT GCCCAGTCAT AGCCGAATAG CCTCTCCACC CAAGCGGCCG
7351 GAGAACCTGC GTGCAATCCA TCTTGTTCAA TCATGCGAAA GGATCCTCAG
7401 CCTGTCTCTT GATCTCCTCT CCAAATGAAA TGAAGTTCCT TATATAGAGG
7451 AAGGGTCTTG CGAAGGATAG TGGGATTGTG CGTCATCCCT TACGTCAGTG
7501 GAGATATCAC ATCAATCCAC TTGCTTTGAA GACGTGGTTG GAACGTCTTC
7551 TTTTTCACG ATGCTCCTCG TGGGTGGGGG TCCATCTTTG GGACCACTGT
7601 CGGCAGAGGC ATCTTGAACG ATAGCCTTTC CTTTATCGCA ATGATGGCAT
7651 TTGTAGGTGC CACCTTCCTT TTCTACTGTC CTTTTGATGA AGTGACAGAT
7701 AGCTGGGCAA TGGAATCCGA GGAGGTTTCC CGATATTACC CTTTGTTGAA
7751 AAGTCTCAAT AGCCCTTTGG TCTTCTGAGA CTGTATCTTT GATATTCTTG
7801 GAGTAGACGA GAGTGTCGTG CTCCACCATG TTGACGAAGA TTTTCTTCTT
7851 GTCATTGAGT CGTAAAAGAC TCTGTATGAA CTGTTCGCCA GTCTTCACGG
7901 CGAGTTCTGT TAGATCCTCG ATCTGAATTT TTGACTCCAT GGCCTTTGAT
7951 TCAGTAGGAA CTACTTTCTT AGAGACTCCA ATCTCTATTA CTTGCCTTGG
8001 TTTATGAAGC AAGCCTTGAA TCGTCCATAC TGGAATAGTA CTTCTGATCT
8051 TGAGAAATAT ATCTTTCTCT GTGTTCTTGA TGCAGTTAGT CCTGAATCTT
8101 TTGACTGCAT CTTTAACCTT CTTGGGAAGG TATTTGATCT CCTGGAGATT
8151 ATTACTCGGG TAGATCGTCT TGATGAGACC TGCCGCGTAG GCCTCTCTAA
8201 CCATCTGTGG GTCAGCATTG TTTCTGAAAT TGAAGAGGCT AATCTTCTCA
8251 TTATCGGTGG TGAACATGGT ATCGTCACCT TCTCCGTCGA ACTTTCTTCC

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FIG. 27e

8301 TAGATCGTAG AGATAGAGAA AGTCGTCCAT GGTGATCTCC GGGGCAAAGG
8351 AGATCCTCTA GCTGATAGTG ACCTTAGGCG ACTTTTGAAC GCGCAATAAT
8401 GGTTTCTGAC GTATGTGCTT AGCTCATTAA ACTCCAGAAA CCCGCGGCTG
8451 AGTGGCTCCT TCAATCGTTG CGGTTCTGTC AGTTCCAAAC GTAAAACGGC
8501 TTGTCCCGCG TCATCGGCGG GGGTCATAAC GTGACTCCCT TAATTCTCCG
8551 CTCATGATCA GCGGCTGAGT GGCTCCTTCA ACGTTGCGGT TCTGTCAGTT
8601 CCAAACGTAA AACGGCTTGT CCCGCGTCAT CGGCGGGGGT CATAACGTGA
8651 CTCCCTTAAT TCTCCGCTCA TGATCAGATT GTCGTTTCCC GCCTTCAGTT
8701 TAAACTATCA GTGTTTGACA GGATATATTG GCGGGTAAAC CTAAGAGAAA
8751 AGAGCGTTTA TTAGAATAAT CGGATATTTA AAAGGGCGTG AAAAGGTTTA
8801 TCCGTTCGTC CATTTGTATG TGCATGCCAA CCACAGG

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FIG. 28a

2726CTGAT GGGCTGCCTG TATCGAGTGG
2751 TGATTTTGTG CCGAGCTGCC GGTCGGGGAG CTGTTGGCTG GCTGGTGGCA
2801 GGATATATTG TGGTGTAAC AAATTGACGC TTAGACAACT TAATAACACA
2851 TTGCGGACGT TTTAATGTA CTGCCATTCG CCATTCAGGC TGCGCAACTG
2901 TTGGGAAGGG CGATCGGTGC GGGCCTCTTC GCTATTACGC CAGCTGGCGA
2951 AAGGGGGATG TGCTGCAAGG CGATTAAGTT GGGTAACGCC AGGGTTTTCC
3001 CAGTCACGAC GTTGTAAC GACGCCAGT GCGaattccc caGATCTAGT
3051 AACATAGATG ACACCGCGCG CGATAATTTA TCCTAGTTTG CGCGCTATAT
3101 TTTGTTTTCT ATCGCGTATT AAATGTATAA TTGCGGGACT CTAATCATAA
3151 AAACCCATCT CATAAATAAC GTCATGCATT ACATGTTAAT TATTACATGC
3201 TTAACGTAAT TCAACAGAAA TTATATGATA ATCATCGCAA GACCGGCAAC
3251 AGGATTCAAT CTTAAGAAAC TTTATTGCCA AATGTTTGAA CGATCTTGCA
3301 CGTTgAgcTc aCGTGGCATA CAGCTAATTA AAAGCATCGG GAAAGGAAGG
3351 AAAATTTATG GCTTTTTCTA ATGCTGCCCC GATTGCTAAC AGACGGTGGT
3401 CTGACCCCGC TAATCCATCA ATTTCCATTC CAACAGGCAA GCGATCAGGT
3451 GTAAGGCAGG CAGGAAGGCT CAACCCAGGT AGGCCTGCGT TGCTGCTTGG
3501 GTCCACATTT CGCACGTAGA TCTTGAAAGT GTTCATCATT GAGCCATTGT
3551 GGATGACTGA CGACTCCTGA CCTATGGCTT TGGCCGCTAA GGGTGCAGTT
3601 GGGAAAAGGA TTGCATCTAA CTGATAGAGT CTGAAGTAAT TCCGATAAGT
3651 GGCCTGGAGC CTTGGCCTGA AGGATTGACG CGCCAGTTCA TATTCATCGT
3701 TGGAAATTTG ATGCCCATCA ATTTGCGCAC TGACAATGTT CGCTACATCG
3751 GGGCTACGtA TaCCTTTGAT AACGTCAGAA AAAGAAACTG TTCCCAAAA
3801 ATCGTCGAGA TACTTTTTTA GAGCGTGTGG AAATTCGTAA AGCGCAATTG
3851 GCAAACCTTG CCCACTATTC AGTTCCTCTA GGTGGGGGAT GTCGGCTTCA
3901 ACAAGGTTA CGCCTCTGTT GGCTAGCAAG CGAATCGTCG TTTCAGCTGC
3951 GAAGGCCACA TCAGCATCAA GGTATCGTA AAAGTAGGTA GTGGGGAGGC
4001 CGATCCGAAG CCCCTTCAGC GGCATGGGTG AAATTTTCGC CGACCGTCCG
4051 GAAATCACCT GGTCGAGGAT TATAACATCG GCTACGCACT GCGCTATGAT

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FIG. 28b

4101 TCCGGCGGTG TCCCGGGTGG GGCTGACCGG TATTATCCGA TCTCTTGGAT
4151 ATCGAGCAAG CGTCGGTCGA AATCCTACTA CGCCACACAG GGCTGCGGGT
4201 AGGCGAACAG ATGCACCGGT ATCGGTGCCT ATGCCGCCTA ACATCAATCG
4251 GCTTGCCACC GCAGCAGCCA CACCACCGCT TGAGCCTCCT GGTATCAGAC
4301 TTGGATTCCA CGGGTTCCGC ACCGCACCGG TGGCATAGTT GTTGCTCGTA
4351 ATTCCAAACG ATAACTCATG CATGTTTCCC GAGGCACCCG GCAGTGCTCC
4401 AGCTGAAAAA AGTCTTTCTG CGACGCGGGA TGGTATCTTT GGCAAGTGGT
4451 TTATCAGCGC CGGAGTAGCA GCGCTTGTAG GAAATATGCC GGTCGCGATG
4501 TTCGCCTTAA AACAGAGTGG AATGCCGCAA AGACCTAATC CGGCGTTTCC
4551 ATGACGATCA ATTTTTTTGG CGCTTCGCCG CAAGCCATCC CAGTCTGTAG
4601 CCAGAAGGGC ATTTAATGGT TTTGCAGCTT GGCAACGCGC TATCAGAGTT
4651 TCTACTAGTT CTAAGCAGGA GTAGTCTTTC CGTCTCAGGC GTTCTAGGGT
4701 TTGTGCTAAC GAGGTAATGG GCACCATggT ggatcctCTA GAGTCTATGT
4751 GTTTTTGTGG GGTATTAATG TTTTATTTTT ATTTGGTTAT GTTTCGGGGT
4801 TTGAATTTCG AGTTGTGAAA ATGCTTGAGG ACGTTTTTAT AGGGTTTTTT
4851 AAGACTGCTT TTTCTGATTT TCTTTGGCAT CTCCTCTCAT TTCGCTATTC
4901 ATTTCCATAT TTGGGAACAT TGAAGTTTAA CCTCTCGCTA TTATTAGTGT
4951 TTTTAGTTTC TCTTAACATT TTTCTCCCTT TTCTAATTTT TGGCTTTGGT
5001 TTCCATGTCT TTGAGAATTT GGCTATTTTG GATTTTAAAA GAATACTCTG
5051 AGAGAGAAAA ATCTATTAGT CACGTGAATA AGTTATAGAC ATAAACTTGA
5101 ATGTATTTCA AGAAGTTCAC ATGCAAACTG ATAAATTTAC AGAACTATTA
5151 TTATTATTAT TATTATTATT ATTAGTTTTT TTGGTTAAAC ACTCTTTTTT
5201 TTTGTCAGAC CTGCAGGCAT GCAAGCTTGG CGTAATCATG GTCATAGCTG
5251 TTTCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC
5301 CGGAAGCATA AAGTGTAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA
5351 CATTAATTGC GTTGCGCTCA CTGCCCCGCTT TCCAGTCGGG AAACCTGTCC
5401 TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG
5451 TATTGGGCGC TATCGGGGGA TCTATAGGGA CTTTAGGTGA TCTGGATTTT

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FIG. 28c

5501 AGTACTGGAT TTTGGTTTTA GGAATTAGAA ATTTTATTGA TAGAAGTATT
5551 TTACAAATAC AAATACATAC TAAGGGTTTC TTATATGCTC AACACGTGAG
5601 CGAAACCCTA TAAGAACCCT AATTCCCTTA TCTGGGAACT ACTCACACAT
5651 VTATTATGGA GAAAATAGAG AGAGATAGAT TTGTAGAGAG AGACTGGTGA
5701 TTTCGAACCC CAGAGTCCCG CTCAGAAGAA CTCGTCAAGA AGGCGATAGA
5751 AGGCGATGCG CTGCGAATCG GGAGCGGCGA TACCGTAAAG CACGAGGAAG
5801 CGGTCAGCCC ATTCGCCGCC AAGCTCTTCA GCAATATCAG GGGTAGCCAA
5851 CGCTATGTCC TGATAGCGGT CCGCCACACC CAGCCGGCCA CAGTCGATGA
5901 ATCCAGAAAA GCGGCCATTT TCCACCATGA TATTCGGCAA GCAGGCATCG
5951 CCATGGGTCA CGACGAGATC CTCGCCGTCG GGCATGCGCG CCTTGAGCCT
6001 GGCGAACAGT TCGGCTGGCG CGAGCCCCTG ATGCTCTTCG TCCAGATCAT
6051 CCTGATCGAC AAGACCGGCT TCCATCCGAG TACGTGCTCG CTCGATGCGA
6101 TGTTTCGCTT GGTGGTCGAA TGGGCAGGTA GCCGGATCAA GCGTATGCAG
6151 CCGCCGCATT GCATCAGCCA TGATGGATAC TTTCTCGGCA GGAGCAAGGT
6201 GAGATGACAG GAGATCCTGC CCCGGCACTT CGCCCAATAG CAGCCAGTCC
6251 CTTCCCGCTT CAGTGACAAC GTCGAGCACA GCTGCGCAAG GAACGCCCGT
6301 CGTGGCCAGC CACGATAGCC GCGCTGCCTC GTCCTGCAGT TCATTCAGGG
6351 CACCGGACAG GTCGGTCTTG AAAAAAGAA CCGGGCGCCC CTGCGCTGAC
6401 AGCCGGAACA CGGCGGCATC AGAGCAGCCG ATTGTCTGTT GTGCCAGTC
6451 ATAGCCGAAT AGCCTCTCCA CCCAAGCGGC CGGAGAACCT GCGTGCAATC
6501 CATCTTGTTT AATCATGCGA AAGGATCCTC AGCCTGTCTC TTGATCTCCT
6551 CTCCAAATGA AATGAACTTC CTTATATAGA GGAAGGGTCT TGCGAAGGAT
6601 AGTGGGATTG TCGGTCATCC CTTACGTCAG TGGAGATATC ACATCAATCC
6651 ACTTGCTTTG AAGACGTGGT TGGAACGTCT TCTTTTCCA CGATGCTCCT
6701 CGTGGGTGGG GGTCCATCTT TGGGACCACT GTCGGCAGAG GCATCTTGAA
6751 CGATAGCCTT TCCTTTATCG CAATGATGGC ATTTGTAGGT GCCACCTTCC
6801 TTTTCTACTG TCCTTTTGAT GAAGTGACAG ATAGCTGGGC AATGGAATCC
6851 GAGGAGGTTT CCCGATATTA CCCTTTGTTG AAAAGTCTCA ATAGCCCTTT

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FIG. 28d

6901 GGTCTTCTGA GACTGTATCT TTGATATTCT TGGAGTAGAC GAGAGTGTCC
6951 TGCTCCACCA TGTGACGAA GATTTTCTTC TTGTCATTGA GTCGTAAAAG
7001 ACTCTGTATG AACTGTTTCG CAGTCTTCAC GGCGAGTTCT GTTAGATCCT
7051 CGATCTGAAT TTTTGACTCC ATGGCCTTTG ATTCAGTAGG AACTACTTTC
7101 TTAGAGACTC CAATCTCTAT TACTTGCCTT GGTTTATGAA GCAAGCCTTG
7151 AATCGTCCAT ACTGGAATAG TACTTCTGAT CTTGAGAAAT ATATCTTTCT
7201 CTGTGTTCTT GATGCAGTTA GTCCTGAATC TTTTGACTGC ATCTTTAACC
7251 TTCTTGGGAA GGTATTTGAT CTCCTGGAGA TTATTACTCG GGTAGATCGT
7301 CTTGATGAGA CCTGCCGCGT AGGCCTCTCT AACCATCTGT GGGTCAGCAT
7351 TCTTTCTGAA ATTGAAGAGG CTAATCTTCT CATTATCGGT GGTGAACATG
7401 GTATCGTCAC CTTCTCCGTC GAACTTTCTT CCTAGATCGT AGAGATAGAG
7451 AAAGTCGTCC ATGGTGATCT CCGGGGCAAA GGAGATCCTC TAGCTGATAG
7501 TGACCTTAGG CGACTTTTGA ACGCGCAATA ATGGTTTCTG ACGTATGTGC
7551 TTAGCTCATT AAACCTCCAGA AACCCGCGGC TGAGTGGCTC CTTCAATCGT
7601 TGCGGTTCTG TCAGTTCCAA ACGTAAAACG GCTTGTCCCG CGTCATCGGC
7651 GGGGGTCATA ACGTGACTCC CTTAATTCTC CGCTCATGAT CAGCGGCTGA
7701 GTGGCTCCTT CAACGTTGCG GTTCTGTCAG TTCCAAACGT AAAACGGCTT
7751 GTCCCGCGTC ATCGGCGGGG GTCATAACGT GACTCCCTTA ATTCTCCGCT
7801 CATGATCAGA TTGTCGTTTC CCGCCTTCAG TTTAAACTAT CAGTGTGTTGA
7851 CAGGATATAT TGGCGGGTAA ACCTAAGAGA AAAGAGCGTT TATTAGAATA
7901 ATCGGATATT TAAAAGGGCG TGAAAAGGTT TATCCGTTTCG TCCATTTGTA
7951 TGTGCATGCC AACCACAGG

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FIG. 29a

2726CTGAT GGGCTGCCTG TATCGAGTGG
2751 TGATTTTGTG CCGAGCTGCC GGTCCGGGAG CTGTTGGCTG GCTGGTGGCA
2801 GGATATATTG TGGTGTAAC AAATTGACGC TTAGACAAC TAATAACACA
2851 TTGCGGACGT TTTAATGTA CTGCCATTGC CCATTCAGGC TCGCAACTG
2901 TTGGGAAGGG CGATCGGTGC GGCCTCTTC GCTATTACGC CAGCTGGCGA
2951 AAGGGGGATG TGCTGCAAGG CGATTAAGTT GGGTAACGCC AGGGTTTTCC
3001 CAGTCACGAC GTTGTAAC GACGGCCAGT GCGAATTCGC AAAACACACC
3051 TAGACTAGAT TTGTTTGTCT AACCCAATTG ATATTAATTA TATATGATTA
3101 ATATTTATAT GTATATGGAT TTGGTTAATG AAATGCATCT GGTTTCATCA
3151 AGAATTATAA AGACACGTGA CATTCAATTA GGATAAGAAA TATGGATGAT
3201 CTCTTTCTCT tTTATTCAGA TAACTAGTAA TTACACATAA CACACAACCT
3251 TGATGCCCCAC ATTATAGTGA TTAGCATGTC ACTATGTGTG CATCCTTTTA
3301 TTTCATACAT TAATTAAGTT GGCCAATCCA GAAGATGGAC AAGTCTAGGT
3351 TGGGTACCGA GTCGGTACC CGATCCGTAG ATTAATTTCT ATTGCGGTAG
3401 TTATATCTCT TCCAAGAGTG TTCGAGAGGA TTGTCCTTTG CCAAATACC
3451 TCCACAATTG TGGATAATTG CACAGACGGC GTTACACGCG GTCTGAATAG
3501 CGCCCTCCAC CCATCCACCG GTGAAGGAAC AACTGCAACC CGCCAAGTAA
3551 ACTCCGGTAT CATTAGTCAT GTCCAGCGCT TGAAAGAAAA GTTCTTCAGA
3601 ATAAAAATCC TCGCCACGCC GGTTGAGTTT GAAAGCTCCC CCGGCATTCT
3651 CGTCTGTAAG CCAATCATGT TGAACAACAT TTTGGTCGTA ATCAGCGCAG
3701 GCGGGAAC TA GATGCTGGG AAACGCCGGG AAAGATCTCG AAATTGCGTC
3751 CCGCAGCAGA CAGAATCGCT CTTTTTTGTC GGAACCGCC AACAGCTTGT
3801 GGGAGTCGTC CTCCCATGTA TAACTGATGA GCACCAGACC TTTACCATTG
3851 GGgTCCTGgG GTCATAGTC CAGGCAGTAC ACTGCTTTTG CGATCCCGTC
3901 CATGAGGACA CAGGACGGGA GGATATGGTC TAACCAAAT TTTCGTTTCA
3951 TCAGCAGGAA GAGTTTTGAC GAGCCTGTCA TATGGCTGTT ATCAACCGCT
4001 TGGTTCAC TG GACGAGAA AATGGTGGTA TCGCATGTCA GACAATGCCT
4051 GAGTTGATA TTTGCGAGTC CAGATGTAAC CACCACCTTA TCATAAAGTT

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FIG. 29b

4101 CAGATATCCC GCTCTTAAGC CTTATCTTTA TTTTGTCTT TTCTTTCTCA
4151 ATCGCCCTGA CTTGAACATG GCGTATACGC TGGCTTACAG ACACACCGTT
4201 AACCACCTGA GTGGCTATTC GACGTGGAAG TTCTGAGATT CCTTCAGAGC
4251 ACATCCGCTG ATTTTCTTCA TATCCGTTTA TGACCAAGCG AAGGATCTCA
4301 ATAAACCCGC TTTCAAAAAC TGGACCAAAC CCGCCAGATC CTATTCCCAT
4351 TAGCTTGAAT AGGTCCCAAT CATGAGGGAA ACTCCATGTT TCACCACCAG
4401 GAGGATGCGT GCCCAGAAAG ATCCTCTCTA TCGCTGAAGA GAAGGACTCC
4451 CTCCCGAAAC GGTTTCAGCCA AGTTTGCCAG GAGTCATGAG CCCGCCTAAT
4501 GTCTCCTGAT TTCAAGGCTT GAGTAATAGC AACAGGCGAA GCCAACACAA
4551 TATCTCCCTC ATGGAAACCG TCCTTCAAGA ACGCACGCCA ACCGCTGTAA
4601 ACGCGATGGA ACAGCTTCGG TGGCTGCTGC CCGGCTTTCC ACATGTATCG
4651 GAGGCCTTGG TAGACCAAGT TAGTGTCGAC TGTGCCGGGA TTTGGGAACG
4701 GCCTCATCGA AGACAGGCCG TACCGCTCGA GGAAGAAAA CAAGCACGAT
4751 GCAGCAGGAG GAAATCGCAT CGCCCCCATT TCGGCCACCA CGCTGGGAGC
4801 GTCCTTGAAA GCATGTGACC AAAGCTTGCC TCCAACCCGA TCACTTGCTT
4851 CATATATTGT AACATCGTCT ACACCAGCAT GAAGCAGTTC GCTTGCCACC
4901 ACGAGTCCGG AAATGCCAGC GCCAATGATC GCCACTTTTG GCTTAGGAAC
4951 ATCTTCCGGA AAGAAGCCGA TCCGTCCACT ATCGGAACAT TGGTCAAAAA
5001 ACGATCTGTA GTCATAGAGC AAGTCAATTG TTGGAAAGGA ACCTGCCGAA
5051 GCCTTCAGCA TCAGGTTTCG ACCATAAGCT ACCATGTCAA ATGGTACGGC
5101 CTCTTCAAGG GTCAGGGTAA ACCCGGTAAC GCGTTTCGTC GTGCCTTTCT
5151 CGTAAGGCGG CATTTGCAGG GTTCGGGAGC GGGCAGTCTT AGAGATTGAA
5201 ATGGCCACGA AATGACTAAG ATCGCTGGAT GCACATCTTT TACCGCTCAG
5251 CAAAAACAGG GTTTGGAGAA GTTGTGCTTC GGAGAAATCC ATACAAAATG
5301 GTGCAAGGTC GATGGCAACG AGACCACTCA CTCTTGCTCG CGCCCAAGGT
5351 TCCGATTCAA GTATCCGCCC CAGAATTTCC TTGCCAACAT AGATGTAAGC
5401 GGAGAGGGCT GCTACCTTCT CACCAGCTGA GATCTCGGGA AAGCGACCGT
5451 CGGCCAGCCT TTTGCAAGCT AACCAGCGC TGCACTCGCC GGAGATTGTA

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FIG. 29c

5501 GTAATCCTCC TTCCCCTAGA AGCTTCACGT TCTGAGAAGG CATCGGAAAC
5551 CCTGCGGTCC AATTCATCAG CCTTATCGAC CATTATCAGA TCCACCATTT
5601 TGGTAGAGAA ATGATCGCAC TGGTTATCAA GGAGAGCTGA AGCTGACATg
5651 GtGgateCCG GGTCGACTgt aattgtaaat agtaattgta atgttgtttg
5701 ttgtttgttg ttgttggtaa ttgttgtaaa aataAGCTGC AGATACGAAA
5751 ACATAAACTT GACGTGACAT TATATAGGGA GGTGGGGGTG GGGGGGGGGG
5801 GGTGTAGAAA AGaGTTTTTG CTGTTATGGa CAACTTTGTT CATACAAAAG
5851 ATGTGAaCTA TTCTTGTTGA ACGGCAATCC CATTTGAATA TAATTCGTGa
5901 GGGTTACTCA CCATTACGGA TAAAaGGGGT TGAATTATTA TTATCTTTTG
5951 GaCTTTGTTT ATAGTAATTT GaCCAATTTT AACAACGGCA AAGATCATAT
6001 TTAGCTCGCG GCTGAAAGTT TTTATATTCA CTAGCCGAAA AAGTGTATGA
6051 AATTTGTATA TTTCTTGAT ACATACAAAA ATATATTTTT TCGGCTATTA
6101 TTTTGAGAGC AACTATACAT TATTATTTTT TCTTTCAAGA AACTTTATTT
6151 TTTGTCGGCC tGGAATTGGT GCACGTTTTT TGGCCCCGCG TTATGATTCA
6201 AAActCTGGC CCGAACAATG GCGTATACAG AATTTTTTCAC AAAACATATT
6251 ATTAACATAT TGTTATGCTA AAATCCTACA AGTTAGAGAT TTATATGATT
6301 TAGTAAGTTT AGAAACGTTA CCTAAAAGTT ATTTTGCATG TTTTGTGGT
6351 TCTCTTAAAG GGAAAAGTAA AATTATTCGT AGATAAAATG AGAATTTTTT
6401 TTAGTTCGGT TCTTTTTAAC TAGAGAAAAT TGAGTCTTAG GTTCAAATAT
6451 GTGAAATTGA AGTACTTTGC TAAAGGGAAA AATAGCACAA AACTCGCTAT
6501 GACGTCAAGT ACATAAGTAT AAAGTACTTT GACTAAATAA AAAAGAAAAA
6551 TTGAAATTCA ATTTACAATT ATATTGTAAG AATATTAAAT AGTGGACCAC
6601 TTTATATACC CAAAGAATAA ACTTGGTTAT TACACTTGAA TGTATGCGAA
6651 GAGTTCAACA AAAGCTACAT GATACTAATA TCCGCAGGGC CTTCTAGAA
6701 ATTATAGCGG GTAGTCACTT TTAAGCCTGT TATTTAAAAT GTATTTATAC
6751 TTTATAATAT ATTTAAAGAT TAACTAATTT ACTCAAATTT TAGGGCATGA
6801 CGTCCTAAAG TTAAACCTC AAAGTTCAAA CTTCAGGACA TCGTGTCTTA
6851 AAGTTCGAAA ATCGTGTCCA GAAATTCGAA TCTTATGTCC TGAATCTTAA

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FIG. 29d

6901 ATTAGCAGTT CAAAAATTTA GGACACTTAG TCCTGAATTT CAAATTAGCA
6951 GCTCAAAAAT TCAGGATATA TATTTAGTCC TTAAGTTTAG ACGAATTGGC
7001 TAATCTTTAA ATACATTATA AATTATGAAT ACATTTTTTA TAGCAAACCT
7051 AAAAGTGGCT ATTGGTGCAG TTCGCCCAGT ACCATCTCAT ATCAGCCCAA
7101 TCATTACAAC CCTCCCATAC AAGAGTTCAA AGTTCAAACA TGCAAGCTAA
7151 CACTATTATA ACAAAGCCT CTCAAATATC TACATCAAGC TTGGCGTAAT
7201 CATGGTCATA GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA
7251 CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG
7301 AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCCAGT
7351 CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG
7401 AGAGGCGGTT TCGGTATTGG GCGCTATCGG GGGATCTATA GGGACTTTAG
7451 GTGATCTGGA TTTTAGTACT GGATTTTGGT TTTAGGAATT AGAAATTTTA
7501 TTGATAGAAG TATTTTACAA ATACAAATAC ATACTAAGGG TTTCTTATAT
7551 GCTCAACACG TGAGCGAAAC CCTATAAGAA CCCTAATTCC CTTATCTGGG
7601 AACTACTCAC ACATVTATTA TGGAGAAAAT AGAGAGAGAT AGATTTGTAG
7651 AGAGAGACTG GTGATTTCGA ACCCCAGAGT CCCGCTCAGA AGAACTCGTC
7701 AAGAAGGCGA TAGAAGGCGA TCGCTGCGA ATCGGGAGCG GCGATACCGT
7751 AAAGCACGAG GAAGCGGTCA GCCCATTCGC CGCCAAGCTC TTCAGCAATA
7801 TCACGGGTAG CCAACGCTAT GTCCTGATAG CGGTCCGCCA CACCCAGCCG
7851 GCCACAGTCG ATGAATCCAG AAAAGCGGCC ATTTTCCACC ATGATATTCC
7901 GCAAGCAGGC ATCGCCATGG GTCACGACGA GATCCTCGCC GTCGGGCATG
7951 CGCGCCTTGA GCCTGGCGAA CAGTTCGGCT GGCGCGAGCC CCTGATGCTC
8001 TTCGTCCAGA TCATCCTGAT CGACAAGACC GGCTTCCATC CGAGTACGTG
8051 CTCGCTCGAT GCGATGTTTC GCTTGGTGGT CGAATGGGCA GGTAGCCGGA
8101 TCAAGCGTAT GCAGCCGCCG CATTGCATCA GCCATGATGG ATACTTTCTC
8151 GGCAGGAGCA AGGTGAGATG ACAGGAGATC CTGCCCCGGC ACTTCGCCCA
8201 ATAGCAGCCA GTCCCTTCCC GCTTCAGTGA CAACGTCGAG CACAGCTGCC
8251 CAAGGAACGC CCGTCGTGGC CAGCCACGAT AGCCGCGCTG CCTCGTCCTG

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FIG. 29e

8301 CAGTTCATTC AGGGCACCGG ACAGGTCGGT CTTGACAAAA AGAACCGGGC
8351 GCCCCTGCGC TGACAGCCGG AACACGGCGG CATCAGAGCA GCCGATTGTC
8401 TGTTGTGCCC AGTCATAGCC GAATAGCCTC TCCACCCAAG CGGCCGGAGA
8451 ACCTGCGTGC AATCCATCTT GTTCAATCAT GCGAAAGGAT CCTCAGCCTG
8501 TCTCTTGATC TCCTCTCCAA ATGAAATGAA CTTCTTTATA TAGAGGAAGG
8551 GTCTTGCGAA GGATAGTGGG ATTGTGCGTC ATCCCTTACG TCAGTGGAGA
8601 TATCACATC. ATCCACTTGC TTTGAAGACG TGGTTGGAAC GTCTTCTTTT
8651 TCCACGATGC TCCTCGTGGG TGGGGGTCCA TCTTTGGGAC CACTGTCGGC
8701 AGAGGCATCT TGAACGATAG CCTTTCCTTT ATCGCAATGA TGGCATTGT
8751 AGGTGCCACC TTCCTTTTCT ACTGTCCTTT TGATGAAGTG ACAGATAGCT
8801 GGGCAATGGA ATCCGAGGAG GTTTCCTGAT ATTACCCTTT GTTGAAAAGT
8851 CTCAATAGCC CTTTGGTCTT CTGAGACTGT ATCTTTGATA TTCTTGGAGT
8901 AGACGAGAGT GTCGTGCTCC ACCATGTTGA CGAAGATTTT CTTCTTGTC
8951 TTGAGTCGTA AAAGACTCTG TATGAACTGT TCGCCAGTCT TCACGGCGAG
9001 TTCTGTTAGA TCCTCGATCT GAATTTTGA CTCCATGGCC TTTGATTCAG
9051 TAGGAACTAC TTTCTTAGAG ACTCCAATCT CTATTACTTG CCTTGGTTTA
9101 TGAAGCAAGC CTTGAATCGT CCATACTGGA ATAGTACTTC TGATCTTGAG
9151 AAATATATCT TTCTCTGTGT TCTTGATGCA GTTAGTCCTG AATCTTTTGA
9201 CTGCATCTTT AACCTTCTTG GGAAGGTATT TGATCTCCTG GAGATTATTA
9251 CTCGGGTAGA TCGTCTTGAT GAGACCTGCC GCGTAGGCCT CTCTAACCAT
9301 CTGTGGGTCA GCATTCTTTC TGAAATTGAA GAGGCTAATC TTCTCATTAT
9351 CGGTGGTGAA CATGGTATCG TCACCTTCTC CGTCGAACTT TCTTCCTAGA
9401 TCGTAGAGAT AGAGAAAGTC GTCCATGGTG ATCTCCGGGG CAAAGGAGAT
9451 CCTCTAGCTG ATAGTGACCT TAGGCGACTT TTGAACGCGC AATAATGGTT
9501 TCTGACGTAT GTGCTTAGCT CATTAACTC CAGAAACCCG CGGCTGAGTG
9551 GCTCCTTCAA TCGTTGCGGT TCTGTCAGTT CCAAACGTAA AACGGCTTGT
9601 CCCGCGTCAT CGGCGGGGGT CATAACGTGA CTCCCTTAAT TCTCCGCTCA
9651 TGATCAGCGG CTGAGTGGCT CTTCAACGT TCGGGTTCTG TCAGTTCCAA

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FIG. 29f

9701 ACGTAAAACG GCTTGTCCCG CGTCATCGGC GGGGGTCATA ACGTGA CTCC
9751 CTTAATTCTC CGCTCATGAT CAGATTGTCG TTTCCCGCCT TCAGTTTAAA
9801 CTATCAGTGT TTGACAGGAT ATATTGGCGG GTAAACCTAA GAGAAAAGAG
9851 CGTTTATTAG AATAATCGGA TATTTAAAAG GCGGTGAAAA GGTTTATCCG
9901 TTCGTCCATT TGTATGTGCA TGCCAACCAC AGG

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FIG. 30a

2726CTGAT GGGCTGCCTG TATCGAGTGG
2751 TGATTTTGTG CCGAGCTGCC GGTCGGGGAG CTGTTGGCTG GCTGGTGGCA
2801 GGATATATTG TGGTGTAAC AAATTGACGC TTAGACAACCT TAATAACACA
2851 TTGCGGACGT TTTTAATGTA CTGCCATTCTG CCATTCAGGC TCGCCAACTG
2901 TTGGGAAGGG CGATCGGTGC GGGCCTCTTC GCTATTACGC CAGCTGGCGA
2951 AAGGGGGATG TGCTGCAAGG CGATTAAGTT GGGTAACGCC AGGGTTTTCC
3001 CAGTCACGAC GTTGTAACAAAC GAGGGCCAGT GCGAATTCTGC AAAACACACC
3051 TAGACTAGAT TTGTTTTGCT AACCCTAATTG ATATTAATTA TATATGATTA
3101 ATATTTATAT GTATATGGAT TTGGTTAATG AAATGCATCT GGTTCATCAA
3151 AGAATTATAA AGACACGTGA CATTCAATTA GGATAAGAAA TATGGATGAT
3201 CTCTTTCTCT tTTATTTCAGA TAACTAGTAA TTACACATAA CACACAACCTT
3251 TGATGCCCCAC ATTATAGTGA TTAGCATGTC ACTATGTGTG CATCCTTTTA
3301 TTTTCATACAT TAATTAAGTT GGCCAATCCA GAAGATGGAC AAGTCTAGGT
3351 TGGGTACCGA GCTCAGCTGG CATAACGCTA ATTAAAAGCA TCGGGAAAGG
3401 AAGGAAAATT TATGGCTTTT TCTAATGCTG CCCCATTGTC TAACAGACGG
3451 TGGTCTGACC CCGCTAATCC ATCAATTTCC ATTCCAACAG GCAAGCGATC
3501 AGGTGTAAGG CAGGCAGGAA GGCTCAACCC AGGTAGGCCT GCGTTGCTGC
3551 TTGGGTCCAC ATTTTCGACG TAGATCTTGA AAGTGTTTAT CATTGAGCCA
3601 TTGTGGATGA CTGACGACTC CTGACCTATG GCTTTGGCCG CTAAGGGTGC
3651 AGTTGGGAAA AGGATTGCAT CTAACGATA GAGTCTGAAG TAATTCCGAT
3701 AAGTGGCCTG GAGCCTTGGC CTGAAGGATT GACGCGCCAG TTCATATTCA
3751 TCGTTGGAAA TTGATGCCC ATCAATTTGC GCACTGACAA TGTTCGCTAC
3801 ATCGGGGCTA CGTATACCTT TGATAACGTC AGAAAAAGAA ACTGTTCCCA
3851 CAAAATCGTC GAGATACTTT TTTAGAGCGT GTGGAAATTC GTAAAGCGCA
3901 ATTGGCAAAC TTGCCCCACT ATTCAGTTCC TCTAGGTGGG GGATGTCGGC
3951 TTCAACAAAG GTTACGCCTC TGTTGGCTAG CAAGCGAATC GTCGTTTCAG
4001 CTGCGAAGGC CACATCAGCA TCAAGGTCAT CGTAAAAGTA GGTAGTGGGG
4051 AGGCCGATCC GAAGCCCCTT CAGCGGCATG GGTGAAATTT TCGCCGACCG

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FIG. 30b

4101 TCCGGAAATC ACCTGGTCGA GGATTATAAC ATCGGCTACG CACTGCGCTA
4151 TGATTCCGGC GGTGTCCCGG GTGGGGCTGA CCGGTATTAT CCGATCTCTT
4201 GGATATCGAG CAAGCGTCGG TCGAAATCCT ACTACGCCAC ACAGGGCTGC
4251 GGGTAGGCGA ACAGATGCAC CGGTATCGGT GCCTATGCCG CCTAACATCA
4301 ATCGGCTTGC CACCGCAGCA GCCACACCAC CGCTTGAGCC TCCTGGTATC
4351 AGACTTGGAT TCCACGGGTT CCGCACCGCA CCGGTGGCAT AGTTGTTGCT
4401 CGTAATTCCA AACGATAACT CATGCATGTT TCCCGAGGCA CCCGGCAGTG
4451 CTCCAGCTGA AAAAAGTCTT TCTGCGACGC GGGATGGTAT CTTTGGCAAG
4501 TGGTTTATCA GCGCCGGAGT AGCAGCGCTT GTAGGAAATA TGCCGGTCGC
4551 GATGTTCGCC TTAAAACAGA GTGGAATGCC GCAAAGACCT AATCCGGCGT
4601 TTCCATGACG ATCAATTTTT TTGGCGCTTC GCCGCAAGCC ATCCCAGTCT
4651 GTAGCCAGAA GGGCATTTAA TGGTTTTGCA GCTTGGCAAC GCGCTATCAG
4701 AGTTTCTACT AGTTCTAAGC AGGAGTAGTC TTTCCGTCTC AGGCGTTCTA
4751 GGGTTTGTGC TAACGAGGTA ATGGGCACCA TggTGGATCC CGGGTCGAct
4801 gtaattgtaa atagtaattg taatgttggt tggtgtttgt tggtgttggt
4851 aattgttgta aaaataAGCT GCAGATACGA AAACATAAAC TTGACGTGAC
4901 ATTATATAGG GAGGTGGGGG TGGGGGGGGG GGGGTGTAGA AAAGaGTTTT
4951 TGCTGTTATG GaCAACTTTG TTCATACAAA AGATGTGAac TATTCTTGTT
5001 GAACGGCAAT CCCATTTGAA TATAATTCGT GaGGGTACT CACCATTACG
5051 GATAAAaGGG GTTGAATTAT TATTATCTTT TGGaCTTTGT TTATAGTAAT
5101 TTGaCCAATT TTAACAACGG CAAAGATCAT ATTTAGCTCG CGGCTGAAAG
5151 TTTTATATT CACTAGCCGA AAAAGTGTAT GAAATTTGTA TATTTCTTGT
5201 ATACATACAA AAATATATTT TTTCGGCTAT TATTTTGAGA GCAACTATAC
5251 ATTATTATTT TTTCTTTCAA GAACTTTAT TTTTGTCCG CctGGAATTG
5301 GTGCACGTTT TCTTGGCCCG CGTTATGATT CAAAACCTCG GCCCGAACAA
5351 TGGCGTATAC AGAATTTTTC ACAAACATA TTATTAACAT ATTGTTATGC
5401 TAAAATCCTA CAAGTTAGAG ATTTATATGA TTTAGTAAGT TTAGAAACGT
5451 TACCTAAAAG TTATTTTGCA TGTTTTGTTG GTTCTCTTAA AGGGAAAAGT

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FIG. 30c

5501 AAAATTATTC GTAGATAAAA TGAGAATTTT TTTTAGTTCG GTTCTTTTAA
5551 ACTAGAGAAA ATTGAGTCTT AGGTTCAAAT ATGTGAAATT GAAGTACTTT
5601 GCTAAAGGGA AAAATAGCAC AAAACTCGCT ATGACGTCAA GTACATAAGT
5651 ATAAAGTACT TTGACTAAAT AAAAAAGAAA AATTGAAATT CAATTTACAA
5701 TTATATTGTA AGAATATTAA ATAGTGGACC ACTTTATATA CCCAAAGAAT
5751 AAAC TTGGTT ATTACACTTG AATGTATGCG AAGAGTTCAA CAAAAGCTAC
5801 ATGATACTAA TATCCGCAGG GCCTTCGTAG AAATTATAGC GGGTAGTCAC
5851 TTTTAAGCCT GTTATTTAAA ATGTATTTAT ACTTTATAAT ATATTTAAAG
5901 ATTA ACTAAT T TACTCAAAT TTTAGGGCAT GACGTCCTAA AGTTTAAACC
5951 TCAAAGTTCA AACTTCAGGA CATCGTGTCC TAAAGTTCGA AAATCGTGTC
6001 CAGAAATTCG AATCTTATGT CCTGAATCTT AAATTAGCAG TTCAAAAATT
6051 TAGGACACTT AGTCCTGAAT TTCAAATTAG CAGCTCAAAA ATTCAGGATA
6101 TATATTTAGT CCTTAAGTTT AGACGAATTG GCTAATCTTT AAATACATTA
6151 TAAATTATGA ATACATTTTT TATAGCAAAC TTAAAAGTGG CTATTGGTGC
6201 AGTTCGCCCC GTACCATCTC ATATCAGCCC AATCATTACA ACCCTCCCAT
6251 ACAAGAGTTC AAAGTTCAAA CATGCAAGCT AACACTATTA TAACAAAaGC
6301 CTCTCAAATA TCTACATCAa GCTTGGCGTA ATCATGGTCA TAGCTGTTTC
6351 CTGTGTGAAA TTGTTATCCG CTCACAATTC CACACAACAT ACGAGCCGGA
6401 AGCATAAAGT GTAAAGCCTG GGGTGCCTAA TGAGTGAGCT AACTCACATT
6451 AATTGCGTTG CGCTCACTGC CCGCTTTCCA GTCGGGAAAC CTGTGCTGCC
6501 AGCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG TTTGCGTATT
6551 GGGCGCTATC GGGGGATCTA TAGGGACTTT AGGTGATCTG GATTTTAGTA
6601 CTGGATTTTG GTTTTAGGAA TTAGAAATTT TATTGATAGA AGTATTTTAC
6651 AAATACAAAT ACATACTAAG GGTTCCTTAT ATGCTCAACA CGTGAGCGAA
6701 ACCCTATAAG AACCCTAATT CCCTTATCTG GGAAC TACTC ACACATVTAT
6751 TATGGAGAAA ATAGAGAGAG ATAGATTTGT AGAGAGAGAC TGGTGATTTC
6801 GAACCCCA GA GTCCCGCTCA GAAGAACTCG TCAAGAAGGC GATAGAAGGC
6851 GATGCGCTGC GAATCGGGAG CCGCGATACC GTAAAGCAGC AGGAAGCGGT

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FIG. 30d

6901 CAGCCCATTG GCCGCCAAGC TCTTCAGCAA TATCACGGGT AGCCAACGCT
6951 ATGTCCTGAT AGCGGTCCGC CACACCCAGC CGGCCACAGT CGATGAATCC
7001 AGAAAAGCGG CCATTTTCCA CCATGATATT CGGCAAGCAG GCATCGCCAT
7051 GGGTCACGAC GAGATCCTCG CCGTCGGGCA TGC GCGCCTT GAGCCTGGCG
7101 AACAGTTCGG CTGGCGCGAG CCCCTGATGC TCTTCGTCCA GATCATCCTG
7151 ATCGACAAGA CCGGCTTCCA TCCGAGTACG TGCTCGCTCG ATGCGATGTT
7201 TCGCTTGGTG GTCGAATGGG CAGGTAGCCG GATCAAGCGT ATGCAGCCGC
7251 CGCATTGCAT CAGCCATGAT GGATACTTTC TCGGCAGGAG CAAGGTGAGA
7301 TGACAGGAGA TCCTGCCCCG GCACTTCGCC CAATAGCAGC CAGTCCCTTC
7351 CCGCTTCAGT GACAACGTCG AGCACAGCTG CGCAAGGAAC GCCCGTCGTG
7401 GCCAGCCACG ATAGCCGCGC TGCCTCGTCC TGCAGTTCAT TCAGGGCACC
7451 GGACAGGTCG GTCTTGACAA AAAGAACCGG GCGCCCCTGC GCTGACAGCC
7501 GGAACACGGC GGCATCAGAG CAGCCGATTG TCTGTTGTGC CCAGTCATAG
7551 CCGAATAGCC TCTCCACCCA AGCGGCCGGA GAACCTGCGT GCAATCCATC
7601 TTGTTCAATC ATGCGAAAGG ATCCTCAGCC TGTCTCTTGA TCTCCTCTCC
7651 AAATGAAATG AACTTCCTTA TATAGAGGAA GGGTCTTGCG AAGGATAGTG
7701 GGATTGTGCG TCATCCCTTA CGTCAGTGGA GATATCACAT CAATCCACTT
7751 GCTTTGAAGA CGTGGTTGGA ACGTCTTCTT TTTCCACGAT GCTCCTCGTG
7801 GGTGGGGGTC CATCTTTGGG ACCACTGTGC GCAGAGGCAT CTTGAACGAT
7851 AGCCTTTCCT TTATCGCAAT GATGGCATTG GTAGGTGCCA CCTTCCTTTT
7901 CTACTGTCCT TTTGATGAAG TGACAGATAG CTGGGCAATG GAATCCGAGG
7951 AGGTTTCCCG ATATTACCCT TTGTTGAAAA GTCTCAATAG CCCTTTGGTC
8001 TTCTGAGACT GTATCTTTGA TATTCTTGGA GTAGACGAGA GTGTCGTGCT
8051 CCACCATGTT GACGAAGATT TTCTTCTTGT CATTGAGTCG TAAAAGACTC
8101 TGTATGAACT GTTCGCCAGT CTTACGGCG AGTTCTGTGA GATCCTCGAT
8151 CTGAATTTTT GACTCCATGG CCTTTGATTC AGTAGGAACT ACTTTCTTAG
8201 AGACTCCAAT CTCTATTACT TGCCTTGGTT TATGAAGCAA GCCTTGAATC
8251 GTCCATACTG GAATAGTACT TCTGATCTTG AGAAATATAT CTTTCTCTGT

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FIG. 30e

8301 GTTCTTGATG CAGTTAGTCC TGAATCTTTT GACTGCATCT TTAACCTTCT
8351 TGGGAAGGTA TTTGATCTCC TGGAGATTAT TACTCGGGTA GATCGTCTTG
8401 ATGAGACCTG CCGCGTAGGC CTCTCTAACC ATCTGTGGGT CAGCATTCTT
8451 TCTGAAATTG AAGAGGCTAA TCTTCTCATT ATCGGTGGTG AACATGGTAT
8501 CGTCACCTTC TCCGTCGAAC TTTCTTCCTA GATCGTAGAG ATAGAGAAAG
8551 TCGTCCATGG TGATCTCCGG GGCAAAGGAG ATCCTCTAGC TGATAGTGAC
8601 CTTAGGCGAC TTTTGAACGC GCAATAATGG TTTCTGACGT ATGTGCTTAG
8651 CTCATTAAAC TCCAGAAACC CGCGGCTGAG TGGCTCCTTC AATCGTTGCG
8701 GTTCTGTCAG TTCCAAACGT AAAACGGCTT GTCCCGCGTC ATCGGCGGGG
8751 GTCATAACGT GACTCCCTTA ATTCTCCGCT CATGATCAGC GGCTGAGTGG
8801 CTCCTTCAAC GTTGCGGTTC TGTCA GTTCC AAACGTAAAA CGGCTTGTCC
8851 CGCGTCATCG GCGGGGGTCA TAACGTGACT CCCTTAATTC TCCGCTCATG
8901 ATCAGATTGT CGTTTCCCGC CTTCA GTTTA AACTATCAGT GTTTGACAGG
8951 ATATATTGGC GGGTAAACCT AAGAGAAAAG AGCGTTTATT AGAATAATCG
9001 GATATTTAAA AGGGCGTGAA AAGGTTTATC CGTTCGTCCA TTTGTATGTG
9051 CATGCCAACC ACAGG

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/08692

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/52 A01H1/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA,A,2 021 643 (PALADIN HYBRIDS INC.) 21 January 1992 see the whole document, in particular pages 28, 35-37 and 65 and the claims. ---	1-16,18, 19,22-24
X	WO,A,92 01799 (PALADIN HYBRIDS INC.) 6 February 1992 see the whole document, in particular pages 5 and 16 and the claims. ---	1-8, 11-16, 19,22-24
X	WO,A,90 08828 (PALADIN HYBRIDS INC.) 9 August 1990 see the whole document, in particular the claims. ---	1-8, 11-16, 19,22-24
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Date of the actual completion of the international search

8 November 1996

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